UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

CHARACTERIZATION OF ALTERNATIVE BIOMARKERS TO CONTROL n-Hexane EXPOSURE AND PREVENT 2,5-HEXANODIONE TOXICITY

Maria Edite da Silva Oliveira Torres

DOUTORAMENTO EM FARMÁCIA

(especialidade em TOXICOLOGIA)

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Tese orientada pela Professora Doutora Maria Luísa Lopes Maio Ribeiro de Andrade Mateus, Professora Auxiliar da Faculdade de Farmácia da Universidade de Lisboa, coorientada pela Professora Doutora Maria Camila Canteiro Batoréu, Professora Associada com Agregação à Faculdade de Farmácia da Universidade de Lisboa, e Professora Doutora Luísa Maria Lima Gonçalves, Professora Associada do Instituto Superior de Ciências da Saúde Egas Moniz, especialmente elaborada para a obtenção do grau de Doutor em FARMÁCIA, especialidade Toxicologia.

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Ao Paulo

Ao João Nuno e à Titinha

Aos meus Pais

"Live as if you were to die tomorrow. Learn as if you were to live forever."

(*Mahatma Gandhi*)

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LIST OF ABBREVIATIONS

μ	Chemical potential
2,5-DMP	2,5-Dimethyl pyrrole
2,5-HD	2,5-Hexanedione
ACGIH	American Conference of Governmental Industrial Hygienists
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer disease
ADP	Adenosine diphosphate
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BEI	Biological Exposure Indices
BLV	Biological Limit Values
BOELV	Binding Occupational Exposure Limit Values
bw	body weight
CDO	Cysteine dioxygenase
CNS	Central nervous system
COSHH	Control of Substances Hazardous to Health Regulations (UK)
CSF	Cerebrospinal fluid
CT	Computed tomography
Cys	Cysteine
delta-ALA	delta-Aminolevulinic acid
DMF	Dimethylformamide
DMPN	Dimethylpyrrole norleucine Nα-acetyl-6-(2,5-dimethylpyrrol-1-yl)norleucine
DMPN NAC	N-acetylcysteine conjugate of DMPN (Nα-acetyl-6-[3-(N-acetylcystein-S-yl)-2,5-dimethylpyrrol-1-yl]norleucine)
DMPN GSH	GSH conjugate of DMPN (2-Acetylamino-6-{3-[2-(4-amino-4-carboxy-butyrylamino)-2- (carboxymethyl-carbamoyl)-ethylsulfanyl]-2,5-dimethyl-pyrrol-1-yl}-hexanoic acid)

EAAC1	Excitatory amino acid carrier 1
EC	European Community
EDTA	Ethylenediaminetetraacetic acid
EI	Electronic impact
ESI	Electrospray ionization
F2-IsoPs	F2-Isoprostanes
FFUL	Faculty of Pharmacy of the University of Lisbon
FOB	Functional observation battery
GC-MS	Gas Chromatography-Mass Spectrometry
GGT	γ-glutamyl transpeptidase
GLPH	Glycogen phosphorylase,
GnRH	Gonadotropin releasing hormone
GSH	Glutathione (γ-glutamyl-L-cysteinyl glycine), in reduced form
GSHt	Total GSH (GSH in reduced form + GSSG)
GSSG	Glutathione in oxidized form
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HNE	4-Hydroxy-2-nonenal
HPLC	High-Performance Liquid Chromatography
HSAB	Hard Soft Acid Base
HSBD	Hazardous Substances Database
HSE	Health and Safety Executive (UK)
i-Med.UL	Research Institute for Medicines and Pharmaceutical Sciences
ip	Intraperitoneal
IS	Internal standard
IsoKs	Isoketals
KP	Kriptopyrrole
LC-MS	Liquid Chromatography–Mass Spectrometry
L-Cys	L-Cysteine
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of detection

LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MAO-B	Monoamine oxidase B
MDA	Malondialdehyde
MEL	Maximum Exposure Limits
MRI	Magnetic Resonance Imaging
MRM	Multiple reactions monitoring
*MRM*1	Transition used for quantification
*MRM*2	Transition used for confirmation
MT	Metallothionein
NAC	Nα-acetyl-L-cysteine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate,
NAL	Nα-acetyl-L-lysine
NF	Neurofilaments,
NFH	Neurofilament heavy chain
NFL	Neurofilament light chain
NFM	Neurofilament middle chain
NIH	National Institute of Health
NH_2	Amino group
NMDA	N-methyl-D-aspartate receptors
NO	Nitric oxide
NOAEL	No Observable Adverse Effect Level
NP	Neuroprostanes
$O_2^{\bullet-}$	Superoxide anion
OEL	Occupational Exposure Limits
OES	Occupational Exposure Standard (UK)
OH•	Hydroxyl
ONOO	Peroxynitrate
OSHA	Occupational Safety and Health Administration (USA)
PD	Parkinson Disease
PEL	Permissible Exposure Limits
PLS	Pyrrole-like substances (pyrrole derivatives)

PNS	Peripheral nervous system
po	*Per os*, by way of the mouth
ROS	Reactive oxygen species
R-NH_2	represents protein amine side chain
RSH	Retained spermatid heads
Rt	Retention time
S	Slope (in regression analysis)
SBD-F	7-Fluorobenzo-2-Oxa-1,3-Diazole-4-Sulfonic Acid
SCOEL	Scientific Committee on Occupational Exposure Limits
SD	Standard deviation
SEM	Standard error of the mean
SIM	Selected ion monitoring
TBP	Tri-n-butylphosphine
TCA	Trichloroacetic acid
TIC	Total ion current
TLV	Threshold Limit Values
TWA	Time-Weighted Average
UK	United Kingdom,
US EPA	United States Environmental Protection Agency
UV-Vis	Ultraviolet-visible Spectrophotometry
VAP	Average path velocity
VCL	Curvilinear velocity
VD	Vascular Dementia
VSL	Straight line velocity
WHO	World Health Organization
γ-GCS,	γ-Glumatyl cysteine synthetase
γGT	γ-Glutamyltransferase
η	Hardness of electrophiles or nucleophiles
σ	Standard error of intercept coefficient (in regression analysis)
σ	Relative softness
ω	Electrophilic index
ω−	Index of nucleophilicity

ABSTRACT

Peripheral biomarkers of cumulative exposure to n-hexane and predictive of its neurotoxicity were investigated through an analytical and a toxicological approach.

Synthesis and identification of the standard pyrroles, dimethylpyrrole norleucine (DMPN), cysteine-pyrrole conjugate (DMPN NAC) and glutathione-pyrrole conjugate (DMPN GSH) was performed by LC-MS/MS and their identification was determined in urine of rats treated with 2,5-hexanedione (2,5-HD), the main neurotoxic metabolite of n-hexane.

Two *in vivo* assays were performed to investigate the toxicological meaning of excretion of DMPN and DMPN NAC and to study the protector/toxicity binomium induced by NAC on 2,5-HD neurotoxicity. Wistar rats were treated with 4 doses of: 400 mg 2,5-HD/kg (Group I), 400 mg 2,5-HD/kg + 200 mg NAC/kg (Group II), 200 mg NAC/kg (Group III) and with saline (Group IV) and the correlation between the changes on motor activity and the levels of urinary DMPN, DMPN NAC, and free 2,5-HD was performed. The results show a significant decrease ($p<0.01$) in urinary DMPN and free 2,5-HD, a significant increase ($p<0.01$) in DMPN NAC excretion and a significant recovery ($p<0.01$) on motor activity in rats co-treated with 2,5-HD+NAC, as compared with rats treated with 2,5-HD alone.

A second in vivo assay, was performed to deeper investigate the interference of NAC on 2,5-HD bioactivity co-treating the rats with 12 doses of 2,5-HD+NAC. Our results confirm that until the 4^{th} dose NAC protects against 2,5-HD neurotoxicity, however, after that dose, there is a progressive increase of rats motor activity dysfunctions. Brain GSH levels were determined and the highest GSH/GSSG ratio was found in co-treated rats, indicating that NAC induces GSH brain recovery, nevertheless, this effect was not accompanied by the recovery of motor activities.

A preliminary study, was implemented in humans, where were identified 2,5-HD and DMPN in urine of an healthy and on a neurodegenerative disease population. Finally, the results suggest that DMPN is a sensitive and specific biomarker of 2,5-HD exposure and NAC interacts with 2,5-HD in a biphasic manner: initially protecting against its neurotoxicity and progressively attenuating this effect, and at higher doses inducing toxicity in rats co-treated with 2,5-HD+NAC.

Keywords: 2,5-HD; Neurotoxicity; NAC; Pyrrole compounds; Urinary biomarkers; Behavioral assays; LC-MS/MS.

RESUMO

O trabalho apresentado nesta tese está inserido na área de Toxicologia, e pretende ser uma contribuição para a selecção de biomarcadores específicos a serem aplicados no controle e prevenção da neurotoxicidade em populações de risco, utilizando uma abordagem analítica e uma toxicológica para o seu desenvolvimento.

Sendo o controlo e prevenção da exposição humana a produtos químicos neurotóxicos uma área importante de pesquisa que certamente contribui para a promoção da saúde pública, pretende-se contribuir para a implementação de medidas de modo a controlar e prevenir a neurotoxicidade induzida pela 2,5-hexanodiona (2,5-HD) estudando alternativas de biomarcadores de exposição e efeito.

Introdução: A exposição crónica ao n-hexano induz neuropatias periféricas classificadas como atrofia axonal no sistema nervoso periférico (SNP) e sistema nervoso central (SNC) tanto em seres humanos como em animais. Estudos *in vivo* e *in vitro* demonstraram que o metabolito responsável pela sua neurotoxicidade é a 2,5-HD. A via metabólica de destoxificação deste solvente também conduz à formação de 4,5-dihidroxi-2-hexanona que é excretada através da urina na forma de conjugado glucorónico. Deste modo, a 2,5-HD e o composto 4,5-dihidroxi-2-hexanona excretados através da urina foram utilizados em análises de rotina como biomarcadores de exposição ocupacional ao n-hexano, sendo a soma dos seus níveis denominada 2,5-HD Total. Em 2001 a Conferência Americana de Higienistas Industriais Governamentais, alterou o Índice de Exposição Biológica ao n-hexano para apenas 2,5-HD, recomendando a determinação de 2,5-HD Livre em vez da Total. Na verdade, a 2,5-HD Livre pode ser considerado um bom biomarcador analítico que nos permite avaliar a quantidade de 2,5-HD que escapa ao processo de destoxificação sendo portanto um melhor indicador preditivo do risco de neurotoxicidade. Estudos mecanísticos mostraram que a administração de 2,5-HD, uma γ-dicetona, a animais experimentais, leva à formação de 2,5-DMP (2,5-dimetilpirrol). A formação destes aductos pirrol é originada através da ligação selectiva da 2,5-HD a grupos ε-amino das lisinas existentes nas proteínas de determinados neurofilamentos do citoesqueleto axonal. Após este passo, ocorrem reacções de autoxidação secundárias do 2,5-DMP que originam intermediários electrofílicos pirrolil-metilo. Estes, por sua vez, reagem com pirróis ainda não autoxidados, formando-se dímeros que produzem ligações cruzadas intra e intermoleculares nas proteínas dos neurofilamentos axonais. De facto, alterações na expressão das proteínas por γ-

dicetonas podem provocar danos a nível da estrutura/membrana e refletir mudanças na síntese de proteínas e/ou na degradação após modificações pós-tradução, possivelmente devido à formação dos aductos com a γ-dicetona e reacções de *crosslinking* (polimerização). Vários autores consideram este conjunto de eventos, o responsável pela indução de disfunções motoras sensoriais, observadas em seres humanos e animais experimentais. Os efeitos neurotóxicos provocados pela exposição ao n-hexano, reflectem alterações comportamentais, que podem ser medidas e utilizadas para rastrear o desenvolvimento progressivo da neurotoxicidade durante a exposição a longo prazo. Estes testes são bastante sensíveis, permitindo detectar alterações progressivas a nível das disfunções motoras em função do tempo de exposição ao n-hexano ou à 2,5-HD.

Além das reacções anteriores, podem ainda ocorrer reacções de dimerização do 2,5-DMP em cadeia, devido à presença de radicais livres. Se estas reacções ocorrerem na presença de nucleófilos biológicos como a N-acetil-L-cisteína (NAC) e glutationa reduzida (GSH), estes antioxidantes podem directa ou indirectamente afectar estas reacções de dimerização, levando à inibição deste processo. Sendo a NAC um precursor da L-cisteína, é uma excelente fonte de grupos sulfidrilo (SH) que estimula e restaura a glutationa reduzida celular (GSH), aumentando o seu aporte no organismo. O papel da NAC para a protecção do SNC foi investigado por Banaclocha (2001), que propôs um mecanismo de acção potencial para a NAC baseado no pressuposto de que a NAC pode atravessar a barreira hemato-encefálica e exercer os seus efeitos benéficos a nível do SNC. Estudos *in vitro* mostraram a formação de aductos pirrólicos secundários estáveis com os grupos sulfidrilo da cisteína, os quais podem inibir a reacção de *crosslinking* pirrol-pirrol. Ao formarem estes aductos pirrólicos secundários, podem provavelmente inibir o processo responsável pela neurotoxicidade da 2,5-HD. No entanto, a formação dos vários aductos pirrólicos pode igualmente ser associada a certas patologias neurodegenerativas, como a doença de Alzheimer, devido à peroxidação lipídica. Assim, torna-se importante o estudo de agentes protectores e/ou antagonistas que possam interferir no mecanismo de formação desses aductos pirrólicos.

Metodologia: Para atingir este objectivo foi realizada inicialmente a síntese de compostos pirrólicos padrão: DMPN (dimetilpirrol norleucina), DMPN NAC (conjugado da DMPN com a N-acetil-L-cisteína, NAC) e DMPN GSH (conjugado da DMPN com a glutationa, GSH). A sua síntese foi confirmada pelo seu estudo por ESI-LC-MS/MS. A identificação destes derivados pirrólicos na urina de ratos expostos à 2,5-HD, pretendeu permitir selecionar um biomarcador

periférico preditivo adequado à neurotoxicidade desta. Assim, a análise dos derivados pirrólicos na urina de ratos, foi feita através de um estudo *in vivo*: ratos tratados, por via ip, com 2,5-HD (400 mg/kg de peso corporal/48-h) permitindo pela primeira vez a identificação dos vários compostos pirrólicos, nomeadamente DMPN, DMPN NAC, DMPN GSH e 2,5-DMP. Procedeu-se também à quantificação de DMPN e DMPN NAC. Considerando que é importante controlar a exposição a agentes neurotóxicos estudando agentes protectores e/ou antagonistas que podem reduzir as patologias devidas à exposição ambiental/ocupacional, a interferência da NAC na neurotoxicidade da 2,5-HD foi avaliada através de outro estudo *in vivo*. Trataram-se ratos Wistar com 4 doses de 400 mg de 2,5-HD/kg de peso corporal/48-h (Grupo I), 400 mg de 2,5-HD/kg de peso corporal/48-h + 200 mg NAC/kg de peso corporal (Grupo II), 200 mg NAC/kg de peso corporal (Grupo III) e com solução salina, via ip (Grupo IV). A 2,5 HD foi administrada aos animais por via ip e a NAC por via oral, na água bebível. Procedeu-se a ensaios comportamentais (*ambulation* e *rearing*) e à análise na urina dos valores urinários de DMPN e DMPN NAC por ESI-LC-MS/MS. Também se efectuou a detecção e análise da 2,5-HD urinária por GC-MS e quantificou-se a 2,5-HD Livre e Total. Para avaliar o binómio protector/toxicidade induzido pela NAC na neurotoxicidade da 2,5-HD procedeu-se a um novo ensaio *in vivo*. Este estudo foi realizado aumentando o tempo de exposição, tratando-se agora os ratos com 12 doses de 2,5-HD. Durante este estudo determinou-se as alterações nos biomarcadores urinários seleccionados, DMPN, DMPN NAC e 2,5-HD Livre, assim como alterações no desempenho da actividade motora. No final da experiência foi estudado o mecanismo de interferência da NAC na neurotoxicidade da 2,5-HD quantificando a L-cisteína e a GSH no cérebro de rato. Foi igualmente investigado se seria possível identificar os biomarcadores seleccionados, 2,5-HD, DMPN e DMPN NAC na urina de uma população saudável e na de pacientes com doenças neurodegenerativas.

Resultados: através do estudo dos padrões sintetizados e utilizando como técnica analítica ESI-LC-MS/MS, foi possível identificar e confirmar pela primeira vez, a presença dos compostos pirrólicos DMPN, DMPN NAC, DMPN GSH e 2,5-DMP na urina dos animais expostos à 2,5-HD (400 mg/kg de peso corporal/48h). Os compostos 2,5-HD e DMPN apresentaram os níveis urinários mais elevados de todos os compostos analisados na urina de ratos expostos. A comparação entre a excreção de DMPN e de DMPN NAC evidenciou a elevada excreção urinária de DMPN relativamente ao DMPN NAC urinário, e a correlação linear entre os níveis urinários de DMPN ou DMPN NAC e as doses administradas. Relativamente ao

estudo *in vivo* em que os ratos foram tratados com 4 doses de 2,5-HD e co-tratados com 2,5-HD+NAC, os resultados mostraram uma diminuição significativa (p<0,01) da DMPN urinária e 2,5-HD Livre e um aumento significativo (p<0,01) na excreção da DMPN NAC e 2,5-HD Total. Verificou-se igualmente uma recuperação significativa (p<0,01) da actividade motora (estudo do andamento na horizontal, e estudo da colocação na posição vertical dos animais) nos ratos do Grupo II, quando comparada com os ratos do Grupo I. Estes resultados indicam que a NAC poderá ter um efeito protector na neurotoxicidade da 2,5-HD. Após o estudo *in vivo* em que se pretendia avaliar o binómio protector/toxicidade induzido pela NAC, na neurotoxicidade da 2,5-HD, aumentado-se para isso o tempo de exposição, e tratando-se agora os ratos com 12 doses de 2,5-HD, os resultados obtidos mostraram que os animais tratados com 2,5-HD (Grupo I) e co-tratados com 2,5-HD+NAC (Grupo II), têm uma diminuição significativa (p<0,01) dos seus pesos corporais e apresentam disfunções significativas na sua actividade motora. Há também uma diminuição significativa (p<0,01) da DMPN urinária e da 2,5-HD Livre. No entanto esta diminuição apenas é verificada até às 8 doses. Verificou-se um aumento significativo (p<0,01) na excreção da DMPN NAC para todas as doses. Assim, os resultados por nós obtidos mostram que depois de 4 doses, há uma redução progressiva do efeito de protecção da NAC e um ligeiro aumento de neurotoxicidade no Grupo II. Relativamente aos níveis de GSSG no cérebro dos animais, os animais dos Grupos II, III e IV apresentam perto de 10, 15,4 e 15,0% do valor de GSH Total, respectivamente, sendo as maiores concentrações de GSSG cerebrais encontradas nos animais tratados apenas com 2,5-HD (Grupo I), aproximadamente 42% da GSH Total. Ambos os Grupos I e II têm um valor inferior para a L-cisteína. A maior proporção de GSH/GSSG é observada no Grupo II, o que demonstra o papel antioxidante da NAC de modo a garantir a redução da GSSG. O Grupo I é o que apresenta menor valor para a razão GSH/GSSG. Sobre o estudo em pessoas saudáveis e pessoas com doenças neurodegenerativas, os nossos resultados mostraram que a 2,5-HD e o composto pirrólico DMPN foram identificados na urina de todos os indivíduos saudáveis assim como na urina dos pacientes. Verificou-se ainda a presença de vestígios de DMPN NAC, mas apenas na urina dos pacientes.

Discussão e Conclusões: sugerimos que a fonte de DMPN nos animais apenas tratados com 2,5-HD é devida à formação de aductos primários amino-pirrol (N-acetil-lisina pirrolizados), e a fonte de conjugados de cisteína é devida à formação de conjugados de cisteína pirrolizados, DMPN NAC, ou seja, aductos tiol-pirrol. Estes dois tipos de aductos podem ocorrer na maioria das proteínas, mas os resultados obtidos confirmam que os aductos de amino-pirrol são formados

em quantidades muito mais elevadas, o que pode ser explicado pelo baixo nível de tióis livres presentes na maior parte das proteínas. Relativamente ao conjugado DMPN GSH e ao composto pirrólico 2,5-DMP estes pirróis foram detectados, mas os seus níveis estavam abaixo do limite de quantificação do método analítico utilizado (52,6 nmol/mL). Em conclusão, DMPN é um forte candidato a ser proposto como um novo biomarcador de exposição à 2,5-HD. No 2º estudo *in vivo* a correlação entre os níveis de 2,5-HD, DMPN, DMPN NAC e DMPN GSH urinários e o desempenho da atividade motora nos ratos dos Grupos I e II foram determinados para clarificar a sua utilização como biomarcadores e investigar o papel da NAC na protecção perante a neurotoxicidade da 2,5-HD. A interferência da NAC na neurotoxicidade da 2,5-HD foi evidenciada por uma diminuição significativa nos níveis de 2,5-HD e DMPN urinários, um aumento significativo dos níveis de DMPN NAC urinários e uma recuperação significativa das atividades motoras neurocomportamentais nos animais do Grupo II, quando comparados com os seus valores em ratos tratados com a 2,5-HD (Grupo I). A escolha dos *endpoints* comportamentais para prever a neurotoxicidade da 2,5-HD foi baseada no seu uso frequente para identificar alterações induzidas por produtos químicos nas funções do sistema nervoso, uma vez que são habitualmente utilizados como indicadores sensíveis da diminuição da actividade motora provocada pela 2,5-HD. Esta constatação preliminar indica que, para as quatro doses administradas de 2,5-HD, a NAC tem um papel protector perante a neurotoxicidade da 2,5-HD. Com base no mecanismo de neurotoxicidade desta molécula, em que esta reage com grupos amino da lisina, formando os aductos pirrólicos, estes resultados podem ser explicados considerando que a formação de DMPN depende directamente da reacção entre a γ-dicetona e o grupo ε-amino da lisina nas proteínas. Efectivamente, os nossos resultados mostraram que em ratos co-tratados com 2,5-HD+NAC (Grupo II) observa-se uma diminuição significativa da 2,5-HD Livre urinária em relação aos seus níveis urinários em ratos do Grupo I. Este facto está de acordo com a recuperação nas funções da atividade motora no Grupo II. Na verdade, é a 2,5-HD Livre que pode atingir os órgãos-alvo e ser responsável pela sua toxicidade. Uma diminuição de níveis de 2,5-HD Livre provavelmente explica a diminuição dos seus níveis urinários assim como dos nívei do DMPN, indicando também a redução da sua neurotoxicidade. A hipótese por nós colocada é que, nos animais do Grupo II, a 2,5-HD Livre pode reagir em parte com os resíduos tiol (SH) de cisteína da NAC ou com grupos sulfidrilo da GSH, antes de atingir os grupos ε-amino de resíduos da lisina nos neurofilamentos axonais. Níveis de 2,5-HD Total encontrados nos animais co-tratados foram mais elevados que os encontrados no Grupo I. Este

resultado pode ser explicado considerando que a 2,5-HD Total, corresponde essencialmente a produtos conjugados de biotransformação, sugerindo que a NAC pode contribuir para a destoxificação da 2,5-HD. Também se verificou que os ratos do Grupo II mostraram um aumento significativo da concentração de DMPN NAC, em comparação com os seus níveis em ratos do Grupo I. Este aumento dos níveis de DMPN NAC pode ser associada à inibição da autoxidação dos pirróis, permanecendo o DMPN livre para se ligar à NAC ou à GSH. De facto, uma vez que a formação dos pirróis não é suficiente para causar neuropatia, sugere-se que a NAC induz o aumento de tióis biológicos, os quais podem contribuir indirectamente para impedir a progressão da neuropatia induzida pela 2,5-HD, por promover a desintoxicação de ROS, reduzindo a probabilidade de autoxidação dos pirróis, a subsequente reacção de *crosslinking* pirrolil-pirrol e o desenvolvimento de neuropatias. Esta hipótese pode explicar a recuperação das atividades motoras nos ratos do Grupo II. Estes resultados sugerem que nas condições estudadas a NAC protege contra a neurotoxicidade da 2,5-HD. No 3º estudo *in vivo* em que se pretendeu estudar o binómio protecção/toxicidade induzido pela NAC na neurotoxicidade da 2,5-HD, esta interferência foi evidenciado por uma diminuição significativa nos níveis urinários da 2,5-HD Livre e do DMPN até às 8 doses no Grupo II em relação ao Grupo I. No entanto, esta diminuição deixou de ser significativa após as 12 doses de tratamento. Verificou-se igualmente um aumento significativo do DMPN NAC urinário no Grupo II em relação ao Grupo I para todas as doses administradas. Os dados comportamentais referidos no estudo anterior foram confirmados porque ocorreu uma recuperação significativa do desempenho da atividade motora no Grupo II, até às 4 doses. No entanto, também foi evidente que esse efeito protector diminuiu progressivamente ao longo do tempo de co-tratamento e a partir da 8ª dose não houve qualquer diferença significativa no desempenho comportamental entre os Grupos I e II. Além disso, para a 12ª dose verificou-se que houve uma inversão do efeito protector da NAC passando provavelmente a produzir efeitos tóxicos. O DMPN NAC, é um composto mais estável do que os outros compostos resultantes da acção da 2,5-HD sobre aminoácidos, peptídeos e proteínas, tendo sido referido recentemente, que devido à sua grande estabilidade, os aductos formados entre o eletrófilo DMPN e os grupos sulfidrilo tiolato podem ser irreversíveis. Neste contexto, sabendo que DMPN NAC, é um composto mais estável do que DMPN, talvez ele se possa acumular no sistema nervoso, induzindo toxicidade retardada nos animais co-tratados do Grupo II. Consequentemente, outra hipótese é que o neurotóxico eletrofílico DMPN, aducte seletivamente a tiolatos-receptor (como o NO) em tríades catalíticas o que resulta em perda de

neurotransmissão, com implicações substanciais para a função da proteína e toxicidade pré-sináptica subsequente. LoPachin e Barber (2006), referem que um grande número de produtos químicos estruturalmente electrófilos causam disfunção sináptica por mecanismos desconhecidos. Grupos tiolato cisteína altamente nucleofílicos, dentro de proteínas sinápticas constituem alvos para neurotóxicos eletrófilos que compartilham a capacidade de aductar ou modificar grupos sulfidrilo nucleofílicos. Assim, neurotóxicos electrófilos podem produzir toxicidade sináptica, modificando esses tióis já que a maioria das proteínas contém resíduos de cisteína. Estes locais sulfidril-tiolato permitem regular a atividade de proteínas, desempenhando um papel direto no processo catalítico enzimático e num amplo espectro de actividades sinápticas, actuando como receptores de óxido nítrico (NO), e outros moduladores redox (H_2O_2) que transitoriamente permitem regular funções enzimáticas. Como conclusão, referimos que a hipótese de que o co-tratamento de ratos com 2,5-HD+NAC tem um efeito oposto dependente da dose: em primeiro lugar, inibindo a formação de ligações cruzadas DMPN em neurofilamentos axionais e protegendo contra a neurotoxicidade da 2,5-HD, em segundo lugar acumulando os aductos formados DMPN NAC (persistentes), que depois de atingir um valor limite induzem neurotoxicidade por mecanismos desconhecidos. Relativamente ao mecanismo de interacção da NAC na neurotoxicidade da 2,5-HD através da determinação dos níveis de GSH do cérebro e de cisteína em ratos tratados com 12 doses de (2,5-HD+NAC), há que referir que o cérebro tem uma potencial capacidade oxidativa, no entanto limitada para compensar o stresse oxidativo. Dentro da célula, as espécies reactivas de oxigénio (ROS) em condições normais estão fisiologicamente presentes em concentrações mínimas, havendo um equilíbrio no estado estacionário entre pró-oxidantes e antioxidantes, o que é necessário para assegurar a eficiência óptima de defesas antioxidantes. No entanto, quando a taxa de geração de radicais livres exceder a capacidade de defesa antioxidante, o stress oxidativo segue com consequentes danos graves para as proteínas e lípidos (Calabrese et al., 2008), e tem sido implicada em mecanismos que conduzem à lesão neuronal em vários estados patológicos do cérebro, incluindo doenças neurodegenerativas. De acordo com Stipanuk et al., (2006), o fígado de mamíferos regula a quantidade de cisteína, mesmo quando a ingestão de aminoácidos de enxofre é deficiente ou excessiva. Ao manter os níveis de cisteína dentro de um intervalo restrito e através da regulação da síntese de glutationa, a qual serve como um reservatório de cisteína, o fígado permite resolver quer a necessidade de ter a cisteína adequada para suportar o metabolismo normal, quer a necessidade de manter os níveis de cisteína abaixo do limite de toxicidade. Este regulação pode em parte explicar os valores mais

baixos de cisteína no cérebro de animais co-tratados, Grupo II e Grupo III, em comparação com os Grupos I e IV. Além disso, o aumento dos níveis de cisteína em tecidos nos grupos (2,5-HD+NAC) e NAC pode levar à sua autoxidação formando cistina e ROS, levando assim à diminuição de conteúdo cerebral de L-cisteína. Estudos clínicos preliminares mostraram alguns benefícios na administração sistémica de NAC em algumas doenças neurológicas degenerativas, sendo que as concentrações de GSH aumentaram no plasma após administração crónica de NAC, podendo aumentar as concentrações de GSH no cérebro o que teria um efeito benéfico a nível do SNC. Vários autores referem que a glutationa encontrada em células de mamíferos existe principalmente na forma reduzida (GSH), sendo a GSSG < 5-10% da GSH Total sob condições fisiológicas normais. Os valores por nós obtidos para os Grupos II, III, e IV estão de acordo com estes valores encontrados por outros autores. Apenas no Grupo I apresentam uma GSSG de 42% da GSH Total. Também é para este grupo que se encontra o valor de GSH/GSSG mais baixo. Como é para o Grupo II que se observa o valor mais elevado de GSH/GSSG, podemos concluir o efectivo papel antioxidadante da NAC na redução da GSSG. Em conclusão, podemos dizer que os nossos resultados não esclarecem como a NAC interfere na taxa de neurotoxicidade da 2,5-HD à medida que a relacão GSH/GSSG aumenta no cérebro em ratos co-tratados com 12 doses, (Grupo II), confirmando a sua função antioxidante no cérebro. No entanto, este aumento não está correlacionada com o aumento observado das disfunções neurocomportamentais nos mesmos ratos (Grupo II), o que significa que suas propriedades antioxidantes após 12 doses de tratamento não provocam efeitos protetores contra a neurotoxicidade da 2,5-HD. Relativamente ao estudo preliminar em adultos saudáveis e com doenças neurodegenerativas a presença de 2,5-HD na urina pode ser explicada pela exposição involuntária ao n-hexano pela população em geral, tanto como um micropoluente ubíquo, como por produção endógena de n-hexano e 2,5-HD, através de reacções do metabolismo e/ou peroxidação lipídica no corpo humano, tal como referido por vários autores. Ambas as hipóteses podem explicar o aparecimento de níveis de 2,5-HD em todas as amostras de urina analisadas. No que diz respeito ao DMPN urinário identificado em todas as amostras de urina, a sua origem é, provavelmente, devida à produção endógena de 2,5-HD e sua subsequente reacção com grupos amino da lisina. No entanto, será efectivamente preciso realizar um novo estudo com uma população saudável maior, onde serão quantificados tanto a 2,5-HD como o DMPN para confirmar estes resultados preliminares. O outro pirrol alvo, DMPN NAC, não foi detectado em amostras de urina controlo. Este resultado é interessante, considerando que nos Grupo III e IV também não foi possível quantificar DMPN NAC. No entanto, em todas as

nove amostras de urina de pacientes foi detectado DMPN NAC em quantidades vestigiais. No entanto, é muito difícil de interpretar a origem de DMPN NAC encontrado em algumas amostras de pacientes com diferentes doenças neurodegenerativas. Estudos do cérebro humano sugerem que o stresse oxidativo tem um papel importante na degeneração neuronal em pacientes mais velhos, e uma convergência de vários fatores (genética, idade e poluição ambiental) inicia uma cascata fisiopatológica comum que envolve stresse oxidativo, peroxidação lipídica e a consequente geração de produtos em regiões cerebrais específicas (LoPachin et al., 2008a,b): por exemplo, no hipocampo e neocortex cerebral nos pacientes com doença de Alzheimer. Assim, o aumento de produtos electrofílicos resultantes da peroxidação lipídica em indivíduos idosos pode produzir sinaptotoxicidade através da sua aducção a proteínas com grupos sulfidrilo nucleofílicos, que têm a função de mediadores intermediários de sinaptotoxicidade em muitas doenças neurodegenerativas. Em conclusão, é provável que, em parte, o DMPN urinário e DMPN NAC, detectados em pacientes possam ser devidos ao aumento da formação de aductos de pirrol com os grupos amino e sulfidrilo de proteínas que podem contribuir para os efeitos neurológicos observados em doenças neurodegenerativas.

Palavras chave: 2,5-HD; Neurotoxicidade; NAC; Compostos pirrólicos; Biomarcadores urinários; Estudos comportamentais; LC-MS/MS.

CHAPTER 1

GENERAL INTRODUCTION

1.1 n-HEXANE

n-Hexane is one of the aliphatics hydrocarbon solvents with significant interest in occupational and environmental health because the repeated human exposure to this solvent may result in neurological disease (Spencer and Schaumburg, 1985). n-hexane (CAS Rnº 110-54-3) is a solvent that has many uses in the chemical and food industries, either in pure form or as a component of commercial hexane (US EPA/635/R-03/012).

Hexane typically is present in mixed solvents products and may be found as pollutant at hazardous waste sites (Spencer and Schaumburg, 1985). The chemical is colorless, low-molecular-weight liquid, relatively volatile, with a characteristic odor, and only slightly soluble water.

It is a common component in lacquers, glues and glue thinner, and is widely used in numerous industrial processes, including cements, inks and adhesives, painting and coating products, laminating plastics, press proofing chemicals and cleaning agents (Fedtke and Bolt, 1986b; DeCaprio, 2000). This solvent is also used for manufacturing and processing leather and furniture, shoe manufacturing, as well as vulcanization of rubber and in dilution and extraction solvents in chemical laboratories (Damstra, 1978; Spencer et al., 1980). It is also used to extract vegetable oils for human consumption, as a benzene substitute in solvent applications (IPCS 122, 1991; Arlien-Søborg, 1992; Huang, 2008; Tshala-Katumbay et al., 2009a) and as a solvent for the extraction of oils from seeds, such as cottonseed and sunflower seed (Stanley, 2002).

The chemical is a minor constituent of crude oil and natural gas and, therefore, represents a variable proportion of different petroleum distillates. For example, n-hexane comprises about 11.6% of unleaded gasoline and about 2% of aviation fuel (ATSDR, 1993a,b; US EPA/635/R-03/012). The presence of n-hexane and its isomers in gasoline and fuels makes this solvent a ubiquitous pollutant to which the general population may be exposed (Brugnone et al., 1991; Perbellini et al., 1993; Bavazzano et al., 1998; Manini et al., 1998).

n-Hexane is rapidly absorbed *via* inhalation in experimental animals and humans, and widely distributed to lipid rich tissues and organs such as the liver, spleen, kidneys, peripheral nerves brain and adrenal glands, with the latter three organs showing the highest levels (DeCaprio, 2000; Huang, 2008).

Chronic exposure to n-hexane is one of the well-known causes of multiple disorders of the nervous system, defined as polyneuropathy, and has been reported in several cases of occupational exposure with inadequate ventilation. Japanese workers of small polyethylene laminating factories, in Nagoya, were reported as the first cases of n-hexane polyneuropathy in the world (Spencer et al., 1980; Iwata et al., 1983; Chang et al., 1993; Takeuchi, 2006). The workers suffered from muscle weakness and impaired sensory function of the hands and feet. Biopsy examination of nerves in leg muscles of the exposed workers showed loss of myelin and degeneration of axons. However, the symptoms of polyneuropathy were reversible with recovery, taking several years, after exposure was ended (Stanley, 2002).

Awareness of this neurotoxic agent and implementation of preventive measures have increased in most industrialized countries, however occasional outbreaks continue (Baldasseroni et al., 2003; Huang, 2008; Neghab et al., 2012). Because of the euphoric effects of n-hexane, teenagers are easily addicted to glue-sniffing (Damstra, 1978; Smith and Albers, 1997). Therefore glue-sniffing neuropathy, also known as huffer's neuropathy, after inhalation of glue vapors became a familiar neurological disease in 70's (Goto et al., 1974; Spencer and Schaumburg, 1975, 1985; Fedtke and Bolt, 1986b).

1.1.1 TOXICOKINETICS OF n-HEXANE (ADME)

1.1.1.1 Absorption

n-Hexane is a volatile lipophilic solvent that may be absorbed through inhalatory, oral, or by dermic route. Occupational exposure is mainly *via* inhalation, representing the skin a minor *via* of n-hexane absorption.

Pulmonary retention in humans varies from 15%-30% over a wide range of exposure concentrations (DeCaprio, 2000). According to Mutti et al., (1984) alveolar retention is about 25% of the inhaled n-hexane corresponding to a lung uptake of about 17%. The post exposure alveolar excretion was about 10% of the total uptake. The molecule of n-hexane crosses easily the alveolar-capillary membrane and enters the bloodstream (Brugnone et al., 1978; Veulemans et al., 1982). Thus, inhalation is the most rapid route of absorption. However, the inhalatory

uptake of n-hexane is dependent of several factors as the alveolar uptake, is greater in obese individuals, as well as during physical exercise, since total uptake of n-hexane increased slightly, due to the higher lung ventilation rate (IPCS 122, 1991).
Previous work also demonstrated that the alveolar air concentrations of n-hexane are correlated with blood concentrations in industrial workers exposed to commercial hexane (Brugnone et al., 1978; Mutti et al., 1984; Perbellini et al., 1986).

In vivo toxicokinetics experiments were performed in male rats (F-344), exposed *via* inhalation to two concentrations (1000 ppm and 10000 ppm) of n-hexane. The absorption was rapid, reaching the plateau levels within 30 min in blood and within 2 h in the other tissues examined (liver, kidney, lungs, testis, brain, and sciatic nerve) (IPCS 122, 1991).

Concerning n-hexane dermal exposure, the absorption is poor. In guinea-pigs Jakobson et al., (1982) and Tsuruta (1982) measured its penetration through excised rat skin and came to a similar conclusion. However, some case reports of percutaneous absorption of n-hexane, referred this solvent as the responsible of peripheral neuropathy in humans (IPCS 122, 1991; DeCaprio, 2000).

Finally it must be considered that, the absorption of n-hexane, or its metabolite 2,5-HD, may be enhanced by co-exposure to other solvents (Iwata et al., 1983a; Ladefoged and Perbellini, 1986; Ahonen and Schimberg, 1988; Ladefoged et al., 1989, 1994; Cardona et al., 1993, 1996).

Several inhalation studies in humans and animals demonstrate the distribution of n-hexane, showing its great affinity to tissues higher lipid content (Perbellini et al., 1986).
In female albino rats exposed to n-hexane levels of 17 000 mg/m^3 (50 000 ppm) for up to 10 h, kidney, adrenal, blood, brain, and spleen levels of hexane reached a steady state after approximately 5 h exposure. However, its concentration in the liver increased linearly with time and did not reach saturation at 10 hours. This finding was related to a direct proportionality between the tissue saturation of n-hexane and the lipid content of the tissues examined, particularly its lipid accumulation in the liver, may be due to its high lipid content, which could account for the non-saturability of liver for hexane. In another study, steady-state n-hexane concentrations were observed in tissues, including liver, from rats exposed to 500 - 10 000 ppm

n-hexane for 6-h. Steady-state concentrations in blood, sciatic nerve, liver, and lung were directly proportional to the exposure level, however there was some evidence of saturation in the kidney, brain, and testis (IPCS 122, 1991; DeCaprio, 2000).
In two separated studies, sciatic nerve was found to have the highest levels, following a 6-h inhalation exposure in rats (Baker and Rickert, 1981; Bus et al., 1982).
n-hexane has also been detected in rat fetuses following inhalation exposure of pregnant females and the concentration in total fetal tissue was similar to that in maternal blood (IPCS 122, 1991; DeCaprio, 2000).

The average half-life for n-hexane in human blood was 1.5-2 hours (US EPA/635/R-03/012) and the thermodynamic distribution coefficient of n-hexane between the human organism and the atmosphere was calculated to be 12 (Filser et al., 1987).

1.1.1.2 Metabolism

Numerous studies in laboratory animals and humans have examined the metabolism of n-hexane. The metabolic pathway, although complex, involve a sequence of hydroxylation and dehydrogenation reactions (Fig. 1.1).

At the first step n-hexane is metabolized through hydroxylation reaction that occurs primarily *via* cytochrome P-450-mediated transformation, while cytoplasmic dehydrogenases reversibly oxidize hydroxylated metabolites to the corresponding ketones. The major metabolites of n-hexane are 2-hexanol, methyl n-butyl ketone, 4,5-dihydroxy-2-hexanone and 2,5-hexanedione (Graham et al., 1999; Manini et al., 1998, 1999; DeCaprio, 2000).

The carbons 1, 2 and 3 of n-hexane are hydroxylated and form hexanols in different proportions (in all species of animals). Detoxification pathway originates 1-hexanol and 3-hexanol that are less toxic metabolites. The former is oxidized to hexanoic acid, which undergoes the usual lipid metabolism.

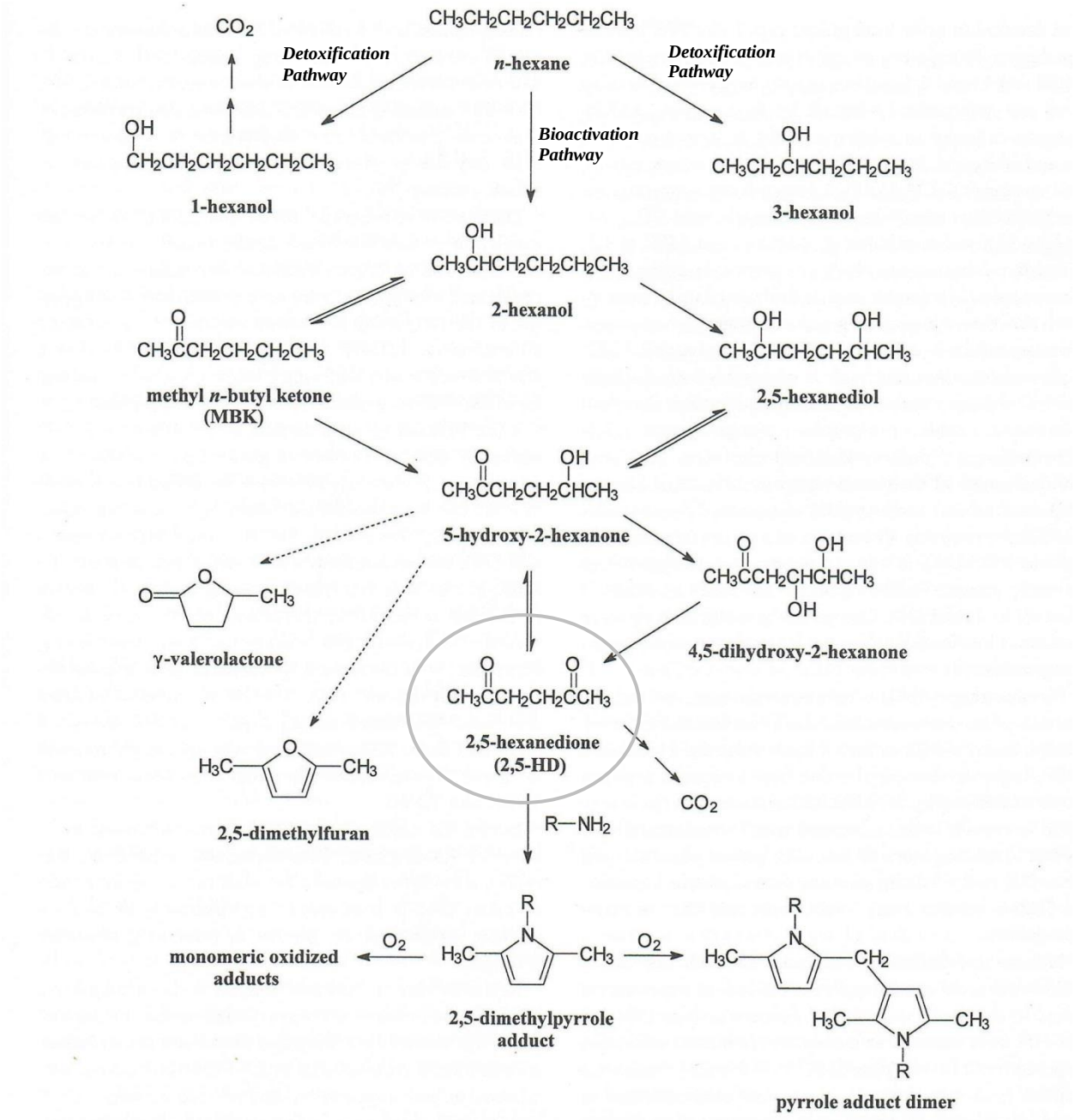


Figure 1.1 Biotransformation and macromolecular binding reactions of n-hexane and 2,5-HD in mammalians systems. Dashed arrows indicate uncertainty in the actual pathway of formation. R-NH_2 represents protein amine side chain. (Adapted from DeCaprio In: Spencer, P.S., Schaumburg H.H., Ludolph A.C. (eds); *Experimental and Clinical Neurotoxicology*. 2nd ed. Oxford University Press, 2000).

Finally, 2-hexanol through a bioactivation pathway is converted in an oxidation reaction, to 2-hexanone and in 2,5-hexanediol through a second hydroxylation. Both of these metabolites are then further metabolized to 5-hydroxy-2-hexanone and 4,5-dihydroxy-2-hexanone. By oxidation of these two compounds 2,5-hexanedione is formed. This diketone is believed to be the major toxic metabolite produced in humans (Perbellini et al., 1980, 1981a; Manini et al., 1998, 1999).
Several P-450 isozyimes are implicated in n-hexane metabolism, with CYP-IIB1 mediating the critical activation pathway from n-hexane to 2-hexanol (Toftgård et al., 1986).

Evidence that the liver is the primary location for the initial hydroxylation step for bioactivation of n-hexane comes from the measurement of hydroxylating activity in isolated microsomes from liver, lung, brain, and skeletal muscles. Microsomes were incubated with n-hexane in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the rates of production of the primary alcohols, 1-, 2-, and 3-hexanol, were compared.
Liver microsomes produced significantly more 2-hexanol than microsomes from lung, brain, and skeletal muscles (soleus and extensor digitorum longus). Similarly, in liver microsomes, 2-hexanol production occurred at a much faster rate than that of 1- or 3-hexanol (Toftgård et al., 1986; Crosbie et al., 1997; US EPA/635/R-03/012).

However, in this process are also generated other secondary metabolites, such as 2,5-dimethylfuran, γ-valerolactone, 2-amino-1-hexanoic acid, pentanone, 2,5-dimethyl-2,3-dihydrofuran and urea. Most of the hydroxylated products are excreted in urine as glucuronides and, possibly, sulfate esters whereas 2-hexanone and 2,5-hexanedione are also present in urine as ‘free’ metabolites (Perbellini et al., 1981b, 1982; Fedtke and Bolt, 1987b; Spencer et al., 2000).

In vivo assays performed in rats demonstrated that 2-hexanol is the major metabolite of n-hexane following inhalation exposure. The authors collected urine samples during and after exposure. The formation of n-hexane metabolites was dependent on exposure concentrations (up to approximately 300 ppm). Formation of 1-, 2-, and 3-hexanol and 2-hexanone was evident during exposure, nevertheless had ceased by 8 hours post exposure. Levels of 2,5-hexanedione and 4,5-dihydroxy-2-hexanone were initially low and the metabolism of n-hexane to these metabolites had ceased by 16 and 40 hours post exposure, respectively. The primary metabolite formed in

rats following inhalation exposure was 2-hexanol (approximately twice all other metabolites observed), followed by 4,5-dihydroxy-2-hexanone. These metabolites together accounted for about 90% of the total metabolites formed. The level of 4,5-dihydroxy-2-hexanone was approximately 10 times higher than 2,5-hexanedione (calculated by subtraction of the concentration of free 2,5-hexanedione measured without acid hydrolysis from the concentration of 2,5-hexanedione measured following complete acid hydrolysis) (Fedtke and Bolt, 1987b).

Bus and collaborators (in 1979) observed the metabolism of n-hexane in the fetus and maternal tissues of pregnant F344 rats and found that 2,5-hexanedione was the major metabolite observed in all maternal tissues evaluated and in the fetus, so they concluded that the metabolism of n-hexane to 2,5-hexanedione and 2-hexanone was rapid and their levels reached peak concentrations in these tissues at 4 hours post exposure (US EPA/635/R-03/012).

1.1.1.3 Excretion

Several human inhalation studies have provided evidence for the elimination of n-hexane and metabolites following occupational and voluntary exposures to n-hexane (Imbriani et al., 1984; Mutti et al., 1984, 1993; Saito et al., 1991; Cardona et al., 1993; Mayan et al., 2001, 2002; Hamelin et al., 2004).
Elimination of n-hexane occurs *via* exhalation of unmetabolized agent, volatile metabolites and CO_2, and by urinary excretion of conjugated and unconjugated metabolites. Some differences among species are apparent in the proportion of various metabolites excreted by different routes of administration (Perbellini et al., 1982).

In rat, elimination from several adult tissues, including sciatic nerve and liver, and from fetal tissue was rapid, however was slower from the kidneys (half-life, 6 h).
In the guinea-pig, biphasic elimination of n-hexane from blood, have half-lives of 0.5 h and 4 h, and from rat blood and brain the elimination was found to be rapid and multiphasic, with initial half-lives of 2 - 4 and 7 min and a subsequent half-life of 1 - 2 h (White et al., 1979; Howd et al., 1982).

In rats, the major urinary metabolites of n-hexane are 2-hexanol, 4,5-dihydroxy-2-hexanone, and 2,5-HD (in that order), although in humans, the latter two compounds predominate (Fedtke and Bolt 1987a,b).

Several human studies support the assertion that 2,5-hexanedione levels in urine are the best estimate of n-hexane exposure in the workplace. However, Hamelin and collaborators (2004) in their study, with five volunteers (three women, two men) that were exposed to n-hexane in an exposure chamber for 2 non-consecutive weeks (7 h/day, 1st week/50 ppm, 2nd week/ 25 ppm), refer that an important fraction of inhaled n-hexane (approximately 73%) was expired unchanged in alveolar air, meaning that only 27% was absorbed by the volunteers. This value agrees with those reported in previous studies, which ranged from 15% to 25% (Brugnone et al., 1978; Veulemans et al., 1982; Mutti et al., 1984). The results also show that there was no significant difference between n-hexane concentrations measured in alveolar air between days of the week, which suggests that n-hexane does not accumulate in blood during a normal week.

Urinary levels of 2,5-HD are used as a routine biomarker of exposure to n-hexane, since it is correlated to that of airborne n-hexane (Perbellini et al., 1981a; IPCS 122, 1991; Andreoli et al., 1998).

1.2 HEXANEDIONE TOXICITY

It is well known that 2,5-HD induces neurotoxic effects. However, the reproductive and the ocular system may be also a target of this toxic metabolite.

1.2.1 NEUROTOXICITY

It is well known that nowadays, it is well stablished, that neuropathies induced by n-hexane are mediated by its main neurotoxic metabolite 2,5-HD.

1.2.1.1 Neurological effects

2,5-Hexanedione induces a central and peripheral distal axonopathy characterized primarily by axonal atrophy in large motor and sensory nerve fibers in experimental animal models (Lehning et al., 1995, 2000; DeCaprio et al., 2009).

An observation consistently made between species, adult and immature members of the same species, and within individual humans and animals, was that longer axons in the PNS and CNS were more vulnerable to the toxic effects of n-hexane and its metabolite, than shorter axons. Observations in humans and investigations in animals have characterized this ataxia and skeletal muscle weakness as a distal sensorimotor neuropathy, specifically a distal axonopathy (dying-back) type (Spencer et al., 1980; Costa, 1996).

LoPachin et al., (2000) conducted an in vivo study to determine which neurological test or combination of tests could provide sufficient functional information to compliment biochemical or morphological endpoints in mechanistic studies of toxic axonopathies.

Rats were exposed to two daily dosing rates (HD, 175 or 400 mg/kg per day, *via po*) and neurological endpoints were determined. Specific tests included observations of spontaneous locomotion in an open field, and measurements of hind limb landing foot splay, forelimb and hind limb grip strength and the hind limb extensor thrust response.

2,5-HD intoxicated rats exhibited hind limb muscle weakness as indicated by a gait abnormality (dropped hocks) and decreases in grip strength and the extensor thrust response. However, hind limb landing foot spread was not affected by 2,5-HD exposure. For this neurotoxicant, gait changes preceded or coincided with alterations in other neurologic indices.

The results suggest that observations of spontaneous behavior in an open field represent a practical approach to assessing temporal development and extent of neurological dysfunction induced by axonopathic toxicants such as 2,5-HD (LoPachin et al., 2000; Mateus et al., 2002).

Peripheral nervous system (PNS) effects

The clinical manifestation of n-hexane is known as central-peripheral distal axonopathy or "dying-back neuropathy" (Huang et al., 1989). The onset is usually subacute or chronic, and the course is progressive with initial symptoms of numbness and burning sensation in the toes and fingers, followed by distal limb muscle weakness (Schaumburg and Spencer, 1976).

The symptoms of polyneuropathy are usually symmetrical (Sanagi et al., 1980; Klassen 2013). Sensory impairments include reduced sensation of temperature, pin-prick, light touch, and vibration as well as position senses in the distal limbs (Schaumburg and Spencer, 1976). The muscle wasting usually occurs in the intrinsic hand and foot muscles, and muscle weakness often involves the extensor and flexor muscles of the legs and forearms. The extensor muscles are usually affected more severely than the flexor muscles in the fore arms and legs (Chang, 1990).

In severely intoxicated patients, proximal muscles may become weak. Tendons reflexes are usually absent in ankle jerks and decreased in knee jerks and biceps (Schaumburg and Spencer, 1976). Autonomic dysfunctions in glue sniffers often include nausea, vomiting, abdominal pain, impotence and skin changes such as coldness, sweating, and exfoliation. Cranial neuropathy includes blurred vision, impaired color vision, retinal or macular changes, decreased corneal reflexes and facial numbness (Dick et al., 2000; Klassen, 2013).

Central nervous system (CNS) effects

The neurotoxic effects on CNS after intoxication by n-hexane or 2,5-HD include headache, sleep disturbance, irritability, mental impairments and spastic gait (DeCaprio, 2000). Acute exposure to high n-hexane concentrations may induce narcosis, euphoria, hallucination, dizziness, giddiness, and headache (Chang, 1987). Severely intoxicated patients may develop respiratory depression, convulsion, coma, and even death. The acute effects are more common in glue sniffers than in industrial workers (Chang, 1987; DeCaprio, 2000).

Hyper reflexes with spasticity are usually observed in polyneuropathic patients several months after recovery of the peripheral neuropathy. The sequelae of spasticity may persist for several years, even after cessation of n-hexane exposure. Extrapyramidal symptoms including rigidity, bradykinesia and tremor are not found. There are no cerebellar signs of nystagmus, tremor, ataxia, or sphincter disturbance (Huang, 2008).

1.2.1.2 Neuropathologic effects

As mentioned above, ultrastructural studies indicate that nervous system toxicity induced by n-hexane and 2,5-HD may be the result of a sequence of events leading to degeneration of the axons in CNS and PNS (Schaumburg and Spencer, 1976; Spencer and Schaumburg, 1977a).

Internodal swelling and axonal atrophy, retraction of myelin from nodes of Ranvier, segmental demyelination, and distal axonal Wallerian-type degeneration are the major neuropathologic lesions (Fig. 1.2) (Graham et al., 1995; DeCaprio et al., 1997; Jortner, 2000; Lehning et al., 2000; LoPachin, 2000.).

The earliest pathological indicator of peripheral nerve axonal degeneration was axonal swelling in the distal nonterminal region of the large myelinated fibers. These axonal swellings appeared first proximal to the nodes of Ranvier and ascended the nerve with further exposure (*i.e.*, facing paranodes and internodal *loci*).

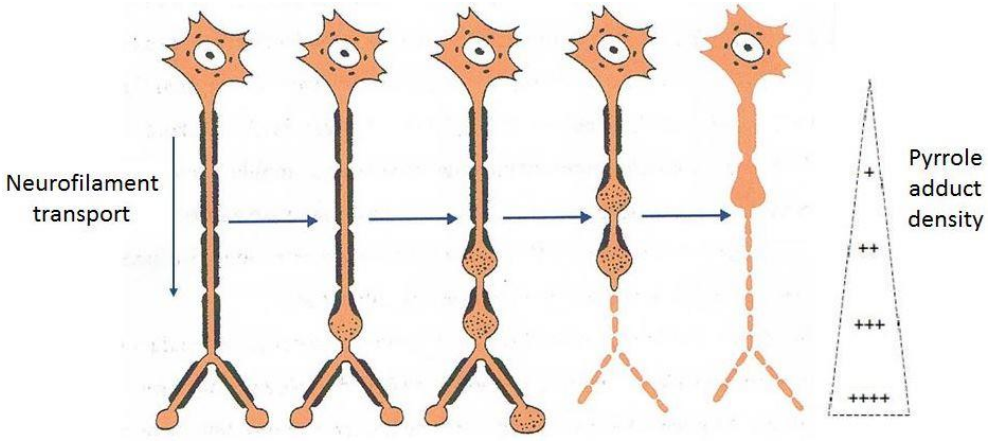


Figure 1.2 The most distally located neurofilaments exhibits the highest pyrrole adducts density, as a consequence of the slow migration of neurofilaments along the axon. This is also the site where the degeneration of the axon is initiated and from where the damage transgresses to more proximal sites (Adapted from: Mateus M.L.; *Avaliação e Prevenção do Risco de Exposição a Misturas com n-Hexano: Parâmetros Bioquímicos e Comportamentais*. Faculdade de Farmácia, Universidade de Lisboa, Lisboa, 2002 and Boelsterli, Urs A., *Mechanistic toxicology: the molecular basis of how chemicals disrupt biological targets*. 1st ed., Taylor & Francis, London, 2003).

Paranodal swelling was accompanied by shrinkage and corrugation of the adjacent distal internode. Paranodal myelin sheaths split and retracted leaving giant axonal swellings near the nodes of Ranvier. Schaumburg and Spencer (1976), and Spencer and Schaumburg (1977a), suggested that Schwann cells may become associated with these denuded regions and remyelinate short segments.

The axonal swellings, that initially occurred proximal to nodes of Ranvier in the most distal internodes of the longest axons, were filled with disorganized masses of NFs. Thus, Graham et al., (1995) postulated that during repeated exposures to n-hexane the resulting metabolite 2,5-HD resulted in progressive derivatization of protein lysyl amino groups to form pyrrolyl adducts. Oxidation of the pyrrole rings to electrophiles lead to increasing levels of cross-linking of NFs during the proximo-distal transport of axoplasm (Tshala-Katumbay et al., 2005; 2009a).

The observations suggested that the constriction of axonal diameter at nodes of Ranvier contributed to the formation of axonal swelling at these locations by presenting obstructions to the transport of the growing masses of NFs.
Additionally, no obstructing masses of NFs could be successfully transported to the synapse for proteolysis. Since the rate of NF transport is 1 mm/day (Griffin et al., 1984; DeCaprio, 2000), axonal length can be seen to determine the period of time during which sufficient NF cross-linking must occur, to produce the threshold masses necessary to occlude transport and result in axonal swellings, secondary myelin retraction and demyelination, and distal axonal degeneration (Tshala-Katumbay et al., 2005).

Consequently, neurofilamentous swellings have been the focus of substantial mechanistic research. However, the pathophysiological relevance of axonal swelling to γ-diketone neurotoxicity has not been demonstrated directly, and in fact, there is now evidence to suggest that swelling is a nonessential phenomenon exclusively related to long-term or low dose-rate intoxication.

The axonal swelling that might not be relevant to the induction of hexacarbon neurotoxicity was first indicated by early studies of Krasavage et al., (1980). These investigators showed that the

frequency of giant axonal swellings in tibial nerve did not correlate with 2,5-HD concentration and was instead related to length of hexacarbon exposure (LoPachin and DeCaprio, 2004).
The relative frequency of swollen axons was inversely related to the serum concentrations of 2,5-HD and to the induction of neurotoxicity. This indicates dissociation between the manifestation of axonal swelling and the development of neurological toxicity.

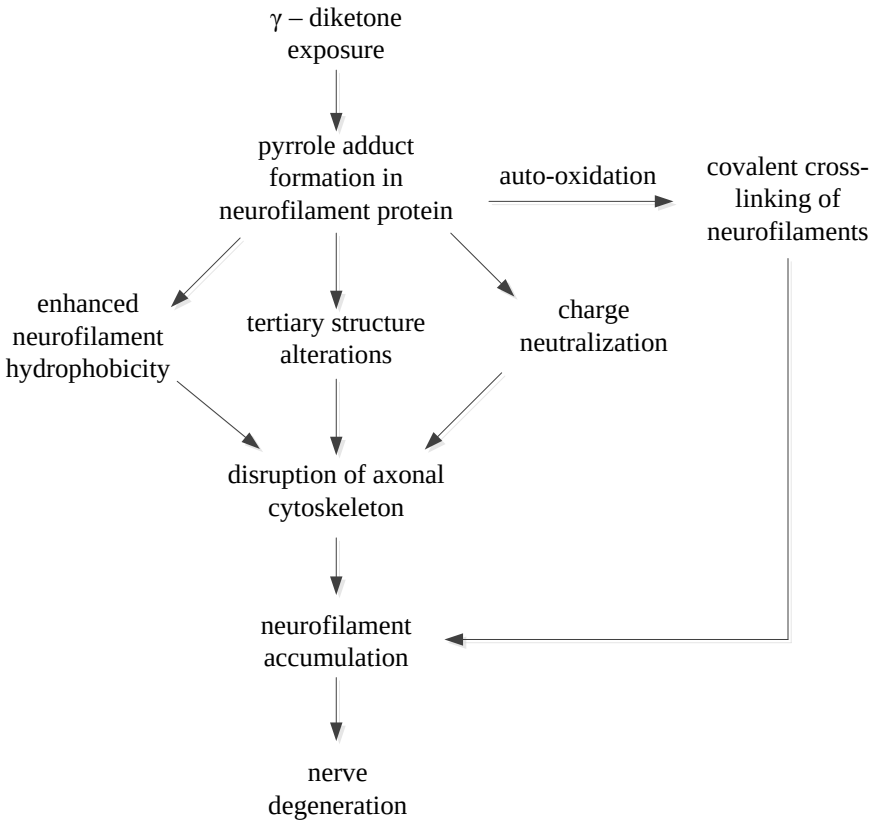


Figure 1.3 Possible putative mechanisms by which nerve fibers (axons) undergo degeneration: result of changes in the proteins, interfering with the ability of mobile NF proteins, interaction with the polymeric cytoskeleton to cause NF accumulation and nerve degeneration. (Adapted from LoPachin and DeCaprio, 2005).

In conclusion, the axon is the site of genesis of γ-diketone axonopathy (Tshala-Katumbay et al., 2005, 2009b), nevertheless the mechanisms by which changes in axonal proteins lead to axonopathy and/or nerve fiber degeneration have yet to be understood (Tshala-Katumbay et al., 2009a). Analytical studies on the γ-diketone susceptibility of selected proteins that are commonly targeted by 2,5-HD and investigations on the time course of proteomic changes as shown in recent studies (Song et al., 2008; Wang et al., 2008) and may provide new insights into

mechanisms by which nerve fibers (axons) undergo degeneration (Fig. 1.3) (Tshala-Katumbay et al., 2009a).

1.2.1.3 Physiological and biochemical effects

Substantial physiological and biochemical studies have explored the mechanism by which 2,5-HD binds to and cross-links proteins. For example, DeCaprio et al., (1982) studied the covalent binding of 2,5-HD to amino acids and polypeptides *in vitro*.

Studies *in vivo* showed that exposure of laboratory animals to 2,5-HD produces axon atrophy in peripheral nervous system (PNS) and central nervous system (CNS) (Lehning et al., 1995, 2000; LoPachin et al., 1994**,** 2003; LoPachin and DeCaprio, 2004).

Changes in the expression of structural/membrane proteins by γ-diketones may reflect changes in protein synthesis and/or degradation after posttranslational modifications possibly by γ-diketo-adduction and crosslinking (polymerization) (DeCaprio, 1985; Spencer et al., 2002; LoPachin and DeCaprio, 2005). The increase in the expression levels of enzymes involved in energy metabolism, except for glycogen phosphorylase (GLPH), possibly represents a stress response involving unbalanced protein turnover associated with protein degradation and/or chemical attack by γ-diketones. Whether the change in the expression levels of these enzymes is accompanied by a production of deleterious species such as free radicals is not known and needs to be investigated (Genova et al., 2004; Tshala-Katumbay et al., 2009a). Reduction in the expression levels of GLPH suggest that impaired glucose metabolism may contribute to the pathogenesis of γ-diketone neurotoxicity.

Atrophy induced changes in axonal cable properties (*e.g.*, decreased unit area) and associated electrophysiological consequences, such as reduced conduction velocity (Yuan et al., 2009), could be responsible for the characteristic neurological deficits that accompany γ-diketone neuropathy.
Their global proteomic approach has allowed the identification of several proteins of interest in the pathogenesis of γ-diketone axonopathy. 2,5-HD reduce the expression level of proteins involved in maintaining the physical integrity of axons or assisting redox/folding mechanisms.

1.2.1.4 Cytotoxic effects

In vitro assays demonstrate that neurons are vulnerable to both n-hexane and 2,5-HD, causing loss of neurons (Selkoe et al., 1978; Cui et al., 2007; Kim et al., 2009).

In vivo assays demonstrated that high doses of 2,5-HD (400mg/kg) could induce significant changes of both anti-apoptosis and pro-apoptosis proteins, which indicates that apoptosis might be involved in 2,5-HD neurotoxicity (Cui et al., 2007). Nevertheless, according to Kim et al., (2009), the effects of lower doses of 2,5-HD, (50 mg/kg), also decreased neurogenesis, increasing ROS formation by approximately 60% relative to controls. These results indicate that 2,5-HD also affects adult hippocampal neurogenesis *via* oxidative stress.

Many evidences demonstrate that 2,5-HD directly induces cell body modifications of neurons (Moretto et al., 1991). Almost all proteins essential for the normal function of axon are synthesized within the perikaryon and subsequently delivered into the axon by axonal transport. According with Cui et al., (2007), neuronal cell body functions critically act in axon maintenance and repair. Since approximately 99% of total neurons exist in CNS, has been reported that 2,5-HD, impairs the spatial memory, mediate some cognitive effect (Carney et al., 2002).

1.2.1.5 Molecular mechanism of 2,5-HD neurotoxicity

Adduct Chemistry: HS/AB Theory

In the field of toxicology, the irreversible covalent interaction of a toxic electrophile and its cognate nucleophilic target is recognized as a basic reaction mediating chemical-induced cell injury. It is now understood that electrophile/nucleophile reactions exhibit a significant degree of selectivity. In that, a given electrophile preferentially reacts with specific nucleophiles of comparable softness or hardness. This selectivity is based on electronic and structural characteristics that constitute the soft and hard classifications of the HSAB theory (Pearson, 1968a,b, 1987).

The significant degree of selectivity that occurs in electrophile ─ nucleophile interactions is predicted by Pearson's Hard and Soft, Acids and Bases theory (HSAB) (Pearson, 1990; Chattaraj, 2001; LoPachin et al., 2008, 2012).
This theory classifies reacting species as either relatively "hard" or "soft", based on polarizability. In this classification, reactive molecules such electrophiles and nucleophiles are classified as either soft (relatively polarizable) or hard (relatively non polarizable). A useful theorem stemming from this principle is that toxic electrophiles react preferentially with biological targets of similar hardness or softness (LoPachin and DeCaprio, 2005).

Relative hardness and softness are clearly important characteristics for covalent bond formation in biological systems. This concept utilizes the inherent electronic characteristic of polarizability (a characteristic of electron distribution in atoms or molecules) to define, for example, reacting electrophiles and nucleophiles as either hard or soft, being polarizability the ease with which electron density can be displaced or delocalized to form new covalent bonds. Toxicant interactions will be either irreversible (covalent adduction) or, at least, slowly reversible (ionic) depending upon the respective chemical characteristics (LoPachin and Barber, 2006).

These HSAB definitions have been successfully applied to chemical-induced toxicity in biological systems. Whereas exceptions exist, most toxic chemicals are electrophiles that produce toxicity by interacting with biological nucleophiles, and therefore, the HSAB concepts should have broad applicability to the field of molecular toxicology in discerning plausible biological targets and molecular mechanisms of toxicity (LoPachin et al., 2012).

The interaction of a soft electrophile with a nucleophile and covalent bond formation is governed and can be described by the shape, property and energy of the respective outermost frontier molecular orbitals. These orbital energies for most chemicals can be calculated, and starting these values and using quantum mechanical models, it is possible to quantify the HSAB parameters. Consequently, the propensity of these chemicals to form adducts can be defined by quantum mechanical parameters: relative softness (σ), hardness (η) of electrophiles or nucleophiles, even as the electrophilic index (ω), the index of nucleophilicity (ω^{-}) and chemical potential (μ). The softness also reflect the rate of adduct formation. On this account is considered the ease with which electron redistribution takes place during covalent bonding and thus, the

softer the electrophile, more readily it will form an adduct by accepting outer shell electrons from a soft nucleophile. Differences in μ, the ability of a nucleophilic species to transfer electrons, or more precisely, electron density to the electrophile, could determine protein targets of these chemicals. Values of μ are independent of pH and reflect the inherent electronic nature of the structural moiety of molecular species (Pearson, 1968a,b; Pearson et al., 1990; Chattaraj et al., 2006; LoPachin et al., 2007b).

These parameters can be used to predict the toxic potential of electrophilic xenobiotic chemicals and facilitate the identification of corresponding nucleophilic molecular sites of action. They are a useful tool in the search for macromolecular targets, converting this information into useful indices of reactivity, and subsequent elucidation of molecular mechanisms of toxicity (Schultz et al., 2006; LoPachin et al., 2008, 2009b, 2012).

According to several authors the neurotoxic n-hexane metabolite, 2,5-HD is a hard electrophile that preferentially forms adducts with harder nucleophiles such as nitrogen atoms on the ε-amino group of lysine residues (Fig. 1.4) (LoPachin et al., 2012). However, according Tshala-Katumbay (2005, 2008, 2009a), aliphatic γ-diketones can react with ε-amino- or sulfyhydryl (SH)-groups of neuroproteins.

Sulfur has a large atomic radius so that its valence electrons are relatively far from the nucleus and, as such, are highly polarizable and soft. Upon ionization of the thiol (*i.e.*, SH→S-), the consequent expansion of the anionic cloud yields the relatively soft (most easily polarizable) thiolate nucleophile and cysteine thiolate could be the softest nucleophile in biological systems (Table 1.1) (Hinson and Roberts, 1992; LoPachin and DeCaprio, 2005; LoPachin et al., 2008a, 2012).

In contrast nitrogens of amino groups on lysine or histidine residues are also nucleophiles nevertheless they have relatively small atomic radii and, accordingly, their electron clouds are less readily distorted. Consequently, such compounds are less polarizable and so much harder nucleophiles (*e.g.*, the primary nitrogen of the amino acid lysine) (LoPachin and DeCaprio, 2005). 2,5-HD is a relatively hard, however weak electrophile that forms lysine adducts very slowly (Tabel 1.1) (DeCaprio et al., 1982; Zhang et al., 2010).

Table 1.1 Specific nucleophilic sites in amino-acid polymers and electrophilic neurotoxicants (metabolites) ordered by increasing hardness. (Adapted from Schultz et al., 2006 and LoPachin et al., 2012).

Specific nucleophilic sites in amino acid sites
1) thiol group of Cys
2) S-atoms of methionine
3) primary amino groups of lysine, arginine and guanine
4) secondary amino-group of histidine
Electrophilic neurotoxicants compounds (or metabolites)
1) 4-hidroxi-2-nonenal
2) acrolein
3) acrilamide
4) 2,5-hexanedione
5) glycidamide

This theory could explain what mechanistic studies showed. This γ-diketone, when administered to experimental animals, can form adducts with numerous proteins, in a selective manner, binding to ε-amino groups of lysine in axonal cytoskeletal proteins and forming 2,5-DMP (2,5-dimethylpyrrole) primary adducts, within specific regions of neurofilaments (NFs) (DeCaprio et al., 1982, 1987; Graham et al., 1982; DeCaprio and Fowke, 1992; DeCaprio et al., 1997; Zhang et al., 2010). The higher affinity exists between the hard nucleophiles, ε-amino groups of lysine in neurofilaments, and the hard electrophile, 2,5-HD (Fig 1.4).

Relative hardness and softness are clearly important characteristics for covalent bond formation in biological systems. However, protein adduction is dependent not only upon the physiochemical nature of the electrophile, also upon the microenvironment of the nucleophilic center, which can vary significantly even among centers of the same elemental type (*e.g.*, sulfur or amino groups) (LoPachin and DeCaprio, 2005).

Thus, nucleophilic reactivity among free sulfhydryl groups on polythiol proteins can be diverse and, consequently, soft electrophilic chemicals will adduct the more reactive thiol groups on a

given protein (Vogel and Nivard, 1994). This diversity in nucleophilic reactivity is a function of both steric and electronic factors mediated primarily by protein tertiary structure (LoPachin and DeCaprio, 2005).

Formation of Pyrrole Compounds

Several studies showed that 2,5-HD could react directly with axonal protein by the formation of N-substituted 2,5-DMP adducts at ε-amine nitrogen of the lysine residues of neurofilaments (Fig. 1.2) (DeCaprio, 1985; Pyle et al., 1992; Graham et al., 1995; Decaprio et al., 1997).

However, only ketones classified as gamma diketones are able to cause this neurotoxic effect. Considerable data support the conclusion that the gamma spacing of the two carbonyl compounds in 2,5-HD is critical to the generation of neurofilamentous axonopathy (DeCaprio, 2000; Tshala-Katumbay et al., 2005; Llorens, 2013).

Secondary oxidation of the pyrrole ring to an electrophile (soft electrophile) reacted with nucleophiles (in neurofilaments) resulting in intra- and intermolecular protein cross-linking (A, B and C in Fig. 1.4), and this was considered to be the determinant event in 2,5-HD neuropathy (Graham et al., 1982; Zhu et al., 1994).

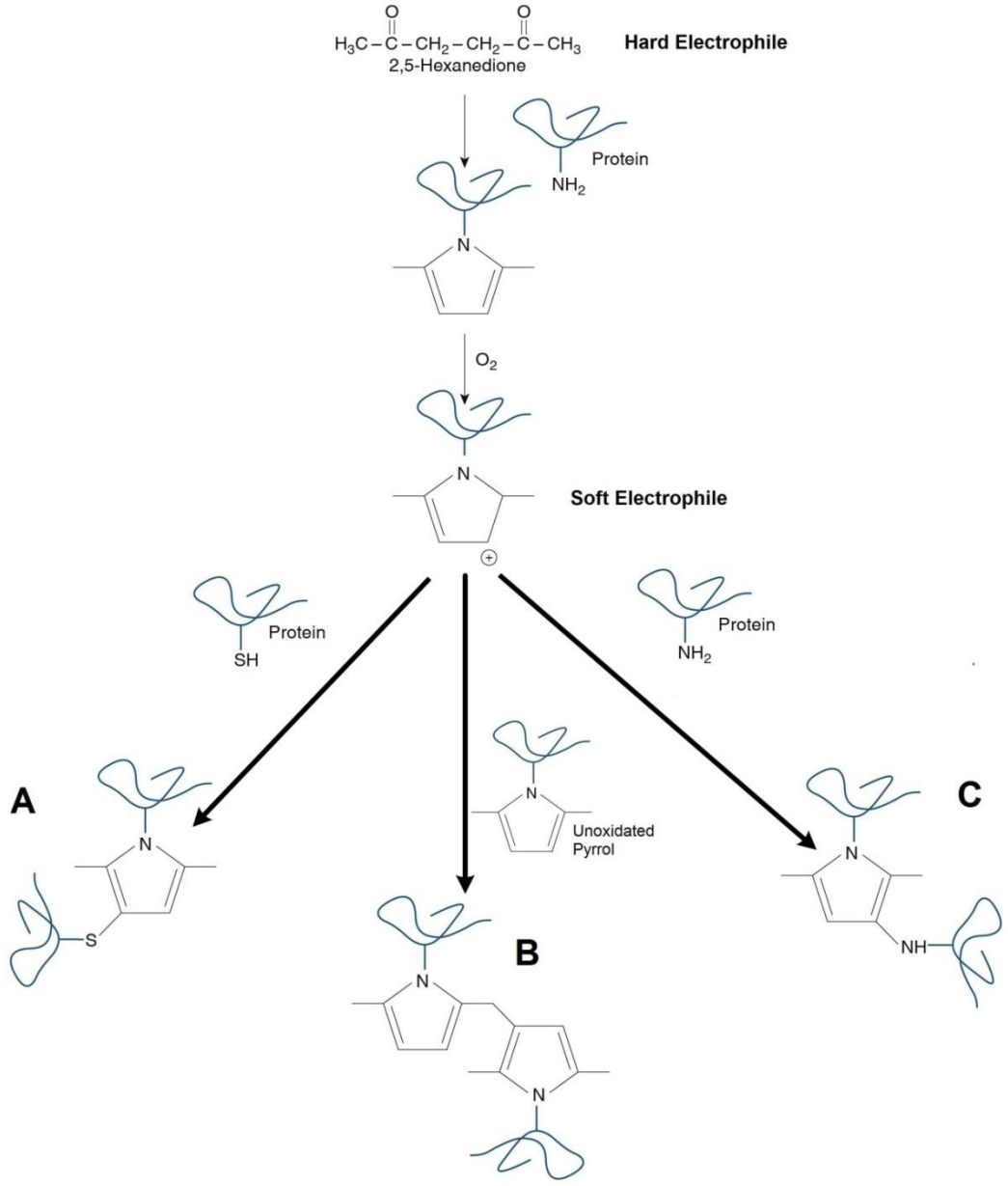


Figure 1.4 Molecular mechanisms of protein cross-linking in the neurofilaments neuropathies. 2,5-Hexanedione is capable of cross-linking proteins. Pyrrole formation from 2,5-HD is followed by oxidation and reaction with adjacent protein nucleophiles (Adapted from Moser et al., In *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 8th ed. McGraw-Hill, New York, 2013, and Mateus M.L.; *Avaliação e Prevenção do Risco de Exposição a Misturas com n-Hexano: Parâmetros Bioquímicos e Comportamentais*. Faculdade de Farmácia, Universidade de Lisboa, Lisboa, 2002).

1.2.2 REPRODUTIVE TOXICS EFFECTS

In vivo n-hexane exposure, beyond to induce peripheral polyneuropathy, result in testicular germ cell loss through the action of its toxic metabolite that causes irreversible testicular atrophy (Krasavage et al., 1980; Boekelheide and Hall, 1991; Allard et al., 1995).

During *in vivo* exposure to 2,5-HD, pyrroles form and accumulate on tissue proteins as a required step in the induction of both testicular and nervous system injuries (DeCaprio et al., 1982; Sayre et al., 1986; Genter et al., 1987). The earliest detectable change was an increase in the polymerization rate of testis tubulin, which occurs primarily in Sertoli cells, the supportive cell of the seminiferous epithelium (Boekelheide and Hall, 1991; Horimoto et al., 2000).
After exposure, Sertoli cell microtubules are altered, followed by a decrease in seminiferous tubule fluid secretion and subsequent germ cell loss, since 2,5-HD targets Sertoli cells, resulting in germ cell apoptosis and testicular atrophy. Considering that these cells have axon-like characteristics, tubulin was considered an attractive molecular target in testicular injury (Boekelheide et al., 2003).

In 2,5-HD-induced testicular atrophy a time delay between administration of the toxicant and the development of testicular injury is evident, and the length and dose rate of exposure determine the magnitude of germ cell loss. 2,5-Hexadione exposure alters testicular microtubule assembly, and the extent of this assembly abnormality correlates with testicular injury as measured by testicular weight loss and histopathology (Boekelheide and Eveleth, 1988; Boekelheide and Hall, 1991). After exposure to 2,5-HD (1% drinking water solution, for 5 weeks), peak germ cell loss occurred 7 weeks later (Boekelheide, 1988; Boekelheide et al., 2003; Moffit et al., 2007), suggesting a time delay between exposure and testicular injury. Germ cell loss persisted up to 75 weeks after 2,5-HD exposure (Boekelheide and Hall, 1991; Allard et al., 1995), indicating that the injury was irreversible.

In the rat, testicular atrophy associated with 2,5-HD exposure can occur at cumulative exposure levels below those that produce clinical neurotoxicity. High-level exposure for relatively brief periods produced testicular injury without clinical evidence of distal polyneuropathy (Boekelheide, 1988), whereas chronic low-level exposure produced clinical evidence of distal

polyneuropathy without testicular injury (Boekelheide and Eveleth, 1988). In fact, the testicular injury was dose-rate sensitive, whereas the extent of nervous system toxicity was related to the total dose over a range of dose-rates (Krasavage et al., 1980).

These tissue-selective pharmacokinetic effects may, in part, explain the predominance of neurotoxicity in human exposures to 2,5-HD precursors. In addition, the clinical manifestations of neurotoxicity are obvious, whereas those of testicular injury are subtle (Boekelheide et al., 2003; Moffit et al., 2007).
According with Boekelheide et al., (2003) a proposed pathogenic sequence for 2,5-HD-induced testicular injury is as follows: 2,5-HD-induced cross-linking of tubulin leads to altered microtubule assembly, which results in altered microtubule-dependent transport, in Sertoli cells. This altered microtubule-dependent transport is manifested as decreased seminiferous tubule fluid formation and a failure to provide adequate support to germ cells causing them to undergo apoptosis.

One of the most common morphological responses of the Sertoli cell to injury is vacuolation. Vacuoles have been described as an early event with 2,5-HD exposure. In some cases the vacuoles are large and discrete, while in others microvacuolation of the basal Sertoli cell cytoplasm is seen. Subsequent to the vacuolation and/or swelling, germ cell degeneration, disorganization or exfoliation is generally seen. The characteristics of the germ cell changes can vary with different toxicants and are related with the nature of the functional disturbance within the Sertoli cell. In the case of 2,5-HD, which require prolonged exposure to elicit toxicity, there is a gradual, multifocal degeneration of germ cells with the frequent formation of multinucleate spermatid aggregates (Creasy, 2001; Moffit et al., 2007; Bryant et al., 2008).

Other reproductive toxics effects that can be observed in the animals are the percentage of motile sperm and sperm count, and have been used as principal parameters for detecting potential effects on spermatozoa. For the males treated with 2,5-HD, which affects sperm indirectly by damage to the Sertoli cell, decreased sperm motion was observed despite the absence of a marked change in testicular weight, epididymal weight or sperm count. Velocity parameters, that directly express sperm motion (swimming speed), average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL), responded sensitively to abnormal sperm motion

in the 2,5-HD studies (Horimoto et al., 2000). Its administration also induced changes in one histopathology endpoint, spermatid head retention. The presence of retained spermatids along the basement compartment of the seminiferous tubule epithelium was identified as a sensitive measure of 2,5-HD-induced testicular injury being retained spermatid heads (RSH) considered as a sensitive histopathological marker of testicular toxicity for subacute 2,5-HD exposure (Moffit et al., 2007; Bryant et al., 2008).

Most commonly used endpoints for male reproductive toxicity are defined as reproductive organs (testis, epididymis and prostate), weight, their morphological changes, and sperm analysis such as sperm count, sperm mobility and viability (EPA, 1996; Creasy, 2001; Yamamoto et al., 2005). It is usually better to study several because generally no individual endpoint is singularly capable of identifying testicular injury (Moffit et al., 2007).

An interesting aspect of 2,5-HD-induced testicular toxicity is that despite the survival of spermatogonia, the lesion is largely irreversible. However, recovery can occur if an agonist is administered (Leuprolide). This appears to function by normalizing the expression of stem cell factor (a growth factor secreted by the Sertoli cell), which is altered by 2,5-HD treatment. This in turn stimulates the dividing spermatogonia to continue through spermatogenesis (Creasy, 2001). Treatment with a gonadotropin releasing hormone (GnRH) agonist can successfully reverse the “irreversible” 2,5-HD-induced testicular atrophy.

1.2.3 RETINA AND VISUAL TOXICS EFFECTS

Animal studies on n-hexane exposure also indicate that 2,5-HD could provoke premature or accelerated deterioration in vision (Spencer et al, 1978; Bäckström, 1998; Carelli et al., 2007). Dose-response color vision loss (acquired dyschromatopsia) and decreases in the contrast sensitivity function occur in workers exposed to n-hexane and adverse effects usually occur only at concentrations above the occupational exposure limits (Mergler et al., 1987, 1991; Moser et al., 2013).

Large percentage of workers in paint manufacturing facilities, adhesive bandage factory and vegetable oils extraction workers, who were exposed to concentrations of solvents that exceeded

the threshold limit values, had acquired dyschromatopsia as assessed by the Lanthony desaturated panel D-15, color vision test (Eguchi et al., 1995; Bull, 2007).

Several workers had no observable clinical abnormalities as assessed by biomicroscopy, funduscopy, and peripheral visual field tests. The color vision losses were mainly blue-yellow losses, although more severe red-green losses were reported (Mergler et al., 1987; Moser et al., 2013). As a rule, acquired blue-yellow losses generally result from lens opacification or outer retinal alterations, whereas red-green losses are associated with inner retinal, retrobular, or central visual pathway alterations (Issever et al., 2002). Moreover, these occupationally exposed workers also exhibited lower contrast sensitivity at intermediate spatial frequencies, which likely reflects alterations in neural function (Lacerda et al., 2011). The data from the Mergler et al., (1987, 1991), studies appear to show gender differences in these adverse visual effects.

A study of female workers, where the Lanthony D-15 desaturated test was used to assess color vision, showed a trend toward increased prevalence of color vision impairment.
Blue-yellow deficits as well as macular changes were observed in workers exposed to n-hexane for 5 to 21 years (Bäckström, 1999; Ballantyne, 2009). These findings correlate with the rod and cone degeneration observed in rats exposed to 2,5-HD (Bäckström et al., 1993; Klaassen, 2013).

Regarding mechanisms, chemically-induced dyschromatopsia has be claimed to be an early sign of more serious neurotoxicity, since it may occurs at levels lower than those producing other neurotoxic effects (Bull, 2007).
Dyschromatopsia associated to organic solvents intoxication has been attributed to maculopathies caused by damage in cone photoreceptors, ganglion cells and optic nerve demielinization (Issever et al., 2002). Blain and Mergler, (1986), suggested that solvent intoxication led to blue-yellow color vision losses and later may develop to red-green color vision loss. It reflects progressive degeneration from outer retina to optic nerve (Eguchi et al., 1995; Lacerda et al., 2011). Grant and Schuman (1993) suggest that this type of visual loss indicates a beginning process of optical neuropathy after exposure to methanol, styrene, toluene, trichloroethylene and organic solvents mixtures.

1.3 CONTROL AND PREVENTION OF OCCUPATIONAL EXPOSURE TO SOLVENTS

During the industrial revolution occupational and industrial diseases became common. There are now many thousands of chemical substances used in industry ranging from metals and other inorganic compounds to complex organic chemicals, and since a man spends on average one-third of his life at work, the environment in that workplace can be a major factor in determining his health.

An estimated 10 million people are potentially exposed to organic solvents in the workplace. Many of the most severe exposures to solvents have occurred as a result of their use in confined spaces with inadequate ventilation (Bruckner et al., 2013).
Although the working environment has improved immeasurably over the last century, some occupations are still hazardous despite legislation and efforts to improve conditions.

Occupational Toxicology is a specific area of Toxicology that was developed to control and prevent the risk of diseases induced by exposure of workers to chemicals. To achieve this goal research was performed to establish environmental and biological threshold limit values of chemicals below which no risk of disease may occur (Grandjean and Landrigan, 2006).
Workplace exposure limits exist for chemical, biological, and physical agents and are recommended as guidelines or promulgated as standards in order to promote worker health and safety. In the most major industrialized nations there is legislation which sets limits on the levels of toxic substances in the workplace.

This involves setting exposure levels based on the results of human epidemiological data and on the results of animal toxicity studies. It requires monitoring of the occupational environment for compliance. The experimental evidence of toxic effects usually includes the determination of a dose-response relationship and no-effect levels in experimental animals.

1.3.1 OCCUPATIONAL EXPOSURE LIMITS

Occupational Exposure Limits (OELs) are established as standards by regulatory agencies or as guidelines by research groups or trade organizations (Topping, 2001; Jakubowski and Trzcinka-Ochocka, 2005; Bolt and Thier, 2006).

The European Commission has established legally enforceable Binding Occupational Exposure Limit Values (BOELV) and Biological Limit Values (BLV) for the protection of health and safety in the workplace. Socioeconomic and technical feasibility factors are also considered in setting these values. The OELs are established based on recommendations of the Scientific Committee on Occupational Exposure Limits (SCOEL), which evaluates published scientific data on hazardous compounds for regulation and provides assessment of exposure limits that "it believes will protect workers from chemical risks" (2006/15/EC; Huang, 2008).

In the United Kingdom compliance with occupational exposure limits (OELs), for airborne substances, is required by the Control of Substances Hazardous to Health Regulations (COSHH), established under the Health and Safety at Work Act 1974. Under the regulations, OELs are used to define adequacy of control by inhalation (HSE, 1997). There are two types, the occupational exposure standard (OES) and the maximum exposure limit (MEL) (Topping et al., 1998).

In the United States, the Occupational Safety and Health Administration (OSHA) promulgate legally enforceable standards known as Permissible Exposure Limits (PELs). These standards are determined and supported by the best scientific evidence available and assure "to the extent feasible that no employee will suffer material impairment of health or functional capacity" with regular exposure "for the period of his working life". OSHA has defined an 8-hour Time-Weighted Average (TWA) PEL as the "employee's average airborne exposure in any 8-hour work shift of a 40-hour work week which shall not be exceeded". The TWA PEL is established as the highest level of exposure to which an employee may be exposed without incurring the risk of adverse health effects (OSHA, 2005).

The American Conference of Governmental Industrial Hygienists (ACGIH) is a trade organization that publishes OELs for chemicals and for physical agents. These take the form of threshold limit values (TLVs) and biological exposure indices (BEIs).

They are frequently revisited and generally reflect current knowledge in occupational toxicology and industrial hygiene (Table 1.2).

Table 1.2 Occupational Exposure Limits (OELs) that are established as standards by regulatory agencies or as guidelines by research groups or trade organizations.

EC	
SCOEL, Scientific Committee on Occupational Exposure Limits,	
OEL	Occupational Exposure Limits
BOELV	Binding Occupational Exposure Limit Values
BLV	Biological Limit Values
USA	
OSHA, Occupational Safety and Health Administration,	
PEL	Permissible Exposure Limits
TWA	Time-Weighted Average
USA	
ACGIH, American Conference of Governmental Industrial Hygienists,	
OEL	Occupational Exposure Limits
TLV	Threshold Limit Value
BEI	Biological Exposure Indices
UK	
COSHH, Control of Substances Hazardous to Health and HSC, Health and Safety Commission,	
OES	Occupational Exposure Standard
MEL	Maximum Exposure Limit

These parameters were developed as guidelines and are not enforceable standards. However, many industries adopt TLVs and BEIs as internal OELs. As stated by the ACGIH, "The TLVs and BEIs represent conditions under which ACGIH believes that nearly all workers may be repeatedly exposed without adverse health effects" (ACGIH, 2003; OSHA, 2005; Huang, 2008).

BEIs are guidelines for biological monitoring and represent levels "most likely to be observed in specimens collected from healthy workers who have been exposed to chemicals to the same extent as workers with inhalation exposure at the Threshold Limit Value" (Mutti, 1999; ACGIH, 2003). BEIs are recommended for analysis of urine, blood, and exhaled air. While hair, fingernails, and other specimens are used in research and forensic toxicology, there are no BEIs for these biological samples.

To protect against the onset of subclinical and clinical neuropathological symptoms of n-hexane exposure, ACGIH proposes a BEI of 0.4 mg/L as an acceptable concentration of free 2,5-HD in urine (non-acid hydrolyzed samples) and a BEI of 5 mg/g creatinine of total 2,5-HD (acid hydrolyzed samples) at the end of shift on the last day of a workweek, Table 1.3 (ACGIH, 2003; Huang, 2008).

Table 1.3 Limit values and biological indices, for n-hexane and 2,5-HD**.**

Substance			**Value**	**Agency**
n-hexane	Europe	TLV-TWA	50 ppm, 180 mg/m^3	SCOEL
	USA	TLV-TWA	50 ppm, 180 mg/m^3	ACGIH, 2005
		PEL	500 ppm	OSHA
2,5-hexanedione	USA	BEI	0,4 mg/L urine [(a)] 5 mg/g creatinine [(b)]	ACGIH, 2005

TLV-TWA, Threshold Limit Values, Time-Weighted Average; SCOEL, Scientific Committee on Occupational Exposure Limits; PEL, Permissible Exposure Limits; ACGIH, American Conference of Governmental Industrial Hygienists; OSHA, Occupational Safety and Health Administration; BEI, Biological Exposure Indices.
[(a)] non-acid hydrolyzed samples;
[(b)] acid hydrolyzed samples.

1.3.2 BIOMONITORING n-HEXANE EXPOSURE

1.3.2.1 Biomarkers as tools in biomonitoring chemicals exposure

Biomonitoring consists of the measurement of toxicants, their metabolites, or molecular signatures of effect in specimens from humans or animals, including urine, blood, feces, exhaled breath, hair, finger or toenails, bronchial lavage, breast milk, and adipose tissue (Thorne, 2013).

These biological indicators are also called biomarkers, and are defined as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (IPCS 222, 2001).

Depending on their use, biomarkers can be classified as markers of exposure, effect and susceptibility and can be applied to get insights on the multi-stage and multi-factor process that is thought to link exposure to long-term outcomes, as shown in Figure 1.5 (NRC, 1987).

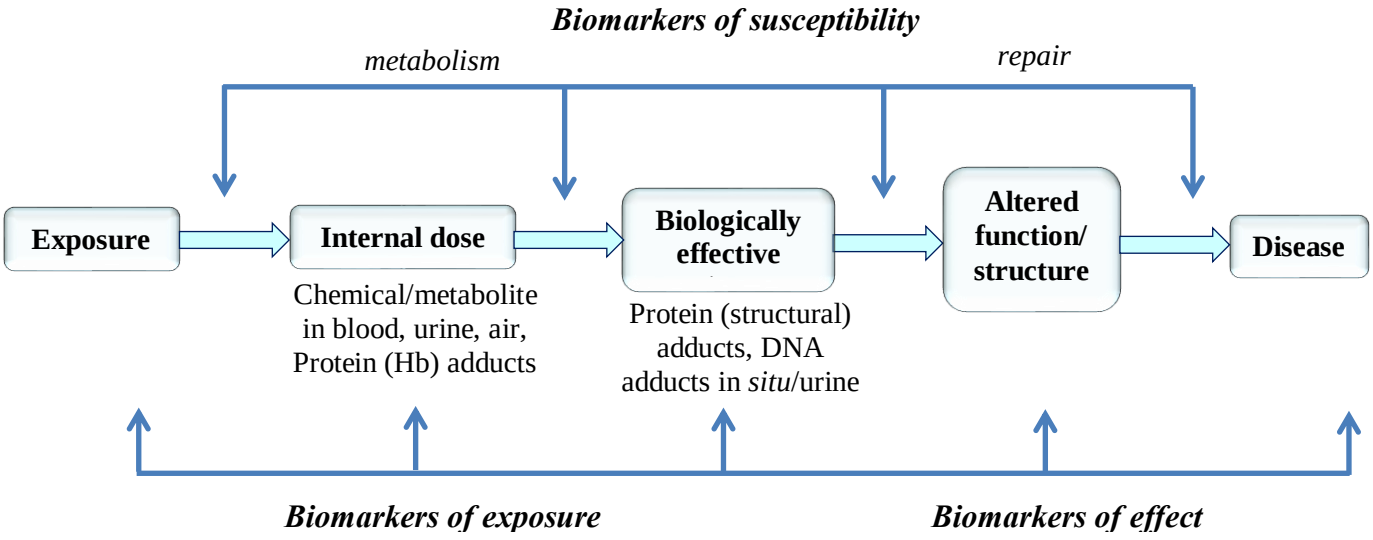


Figure 1.5 Simplified flow chart with classes of biological markers: continuum of events between exposure and disease. Solid horizontal arrows indicate progression, if it occurs. Individual susceptibility influences the rates of progression. Biological markers represent a continuum of changes, and the classification of change may not always be distinct. (Adapted from: NRC, 1987; Manini et al., 2007).

An ideal biomarker of exposure should be specific for the exposure of interest, detectable in trace levels, measurable by non-invasive techniques, inexpensive, associated with prior exposure and provide a predictive value to a specific health status.
In occupational toxicology, the major focus of interest has long been, to clarify if biomarkers of exposure can provide reliable measurements of internal doses, which are useful to assess dose–response relationships (Manini et al., 2004).
External exposure is the sum of the xenobiotic material presented to an organism, whereas internal dose is the amount of the xenobiotic compound that is actually absorbed into the organism. Biological markers of internal dose may include pharmacokinetic data, such as half-life, circulating peak, or cumulative dose. Biologically effective dose is the amount of material interacting with critical subcellular, cellular, and tissue targets or with an established surrogate (NCR, 1987).

In fact, when a dose–response relationship is known, an appropriate biomarker of internal dose may be sufficient to assess the risk of adverse effects. Urine and blood are the most used biological samples for the determination of parent compounds and/or their metabolites and for its non-invasiveness, however, when possible, urine is preferable due to its non-invasiveness. Blood is needed for the determination of macromolecular adducts, which are considered biomarkers of biologically effective dose.

A biological marker of an effect or response can be any change that is qualitatively or quantitatively predictive of health impairment or potential impairment resulting from exposure. An effect is defined as "an health impairment or (by general consensus) recognized disease; an early precursor of a disease process that indicates a potential for impairment of health; or an event peripheral to any disease process correlated with it and thus predictive of development of impaired health".

It is important to realize, however, that these markers represent points on a continuum evolution, whose boundaries may change as knowledge increases.
Early biological responses may include alterations in the functions of the target tissue shortly after exposure. Organs or tissues that are not directly involved in the disease process may also exhibit a response proportional to the biologically effective dose.

The ideal biomarkers should be predictive of effect, should be detected early and be able to show adverse effects before they are irreversible. They are particularly useful in the evaluation of progressive diseases ranging symptoms that occur sometime after the initial exposure to chemicals. In such cases, traditional early warning symptoms of developing disease may be lacking. At the same time, the disease, once clinically apparent, may be essentially irreversible.

Those are the most studied biomarkers and they include modifications in some parameters of blood composition, alterations of specific enzyme activities, the appearance of DNA or protein adducts, localized mRNA and protein increases and the appearance of specific antibodies (autoantibodies) against a xenobiotic or a particular cellular fraction (Gil and Pla, 2001).

1.3.2.2 Biomarkers of Exposure

Exposure to n-hexane in occupationally exposed workers has been mostly assessed by determination of urinary levels of the toxic metabolite 2,5-hexanedione (Kawai et al., 1991b; Saito et al., 1991). However, this measurement reflects only recent exposure, in the order of a few hours to a few days, due to its rapid elimination.

2,5-Hexanedione which metabolic pathway of detoxification leads to the formation of 4,5-dihydroxy-2-hexanone is also excreted in urine as a glucuronide conjugated form (Fedtke and Bolt 1987a,b; DeCaprio et al., 1983; Zhu et al., 1993). Therefore, urinary levels of total 2,5-HD (free 2,5-HD + 4,5-dihydroxi-2-hexanone + 5-hydroxy-2-hexanone) have been used in routine analysis as a biomarker of occupational exposure to n-hexane (Perbellini et al., 1990a). Furthermore, the amount of 2,5-HD detected in urine varies more than tenfold, depending on the pretreatment of urine (at acid pH) prior to analysis (Fedtke and Bolt 1986a,b, 1987a). In 2001, the American Conference of Governmental Industrial Hygienists modified the biological exposure index (BEI) for n-hexane and suggested measuring free urinary 2,5-HD (without hydrolysis), as 0.4 mg/g creatinine (or 3.5µmol/L), instead of total 2,5-HD (acid hydrolysis), corresponding to a Threshold Weight Average, (TWA), of 50 ppm (American Conference of Governmental Industrial Hygienists, 2004). This modification is justified, since the treatment of urine with acid hydrolysis converts other n-hexane metabolites (4,5-dihydroxy-2-hexanone and

5-hydroxy-2-hexanone) into 2,5-HD. These other n-hexane metabolites are not neurotoxic and are conjugated to glucuronic acid and, therefore, easily excreted in urine. Moreover, the hydrolysis of urine is not a necessary step in the measurement of 2,5-HD, since it is excreted unbound in urine (Fedtke and Bolt 1987b; Manini et al., 1999).
In fact, "free" 2,5-HD is a good analytical and biological biomarker of n-hexane exposure, that may indicate the amount of 2,5-HD that escapes to the detoxification process, and might provoke, neurotoxicity, being a better predictor of the neurotoxic risk than the conjugated metabolites that are rapidly excreted in urine (Manini et al., 1999, 2004, 2006; Mateus et al., 2001).

According to Manini et al. (2006), the reappraisal of n-hexane metabolism allowed to draw important conclusions in occupational field: "free" 2,5-HD is both suitable from an analytical point of view and meaningful for biological monitoring purposes, since the neurotoxic risk arising from conjugated metabolites is negligible. Thus, since "free" 2,5-HD corresponds to the main metabolite responsible for the neurotoxicity of n-hexane, its determination is a good indicator of neurotoxicity.
However, there are limitations in its analytical determination resulting from the low levels found in individuals exposed (false negatives) and because this compound was detected in urinary concentrations, for total 2,5-HD, in the order 0.2 to 0.8 mg/L, in individuals not exposed to n-hexane (Fedtke and Bolt 1986b; Bavazzano et al., 1998). This 2,5-HD, also called endogenous, could be due to environmental pollutants, to the process of lipid peroxidation, or to intermediates resulting from the metabolism of carbohydrates in the body (Filser et al., 1983; Perbellini et al., 1993; Manini et al., 1998).

Other biomarkers could alternatively be used for the biological monitoring of n-hexane, for instance, the measurement of n-hexane in blood can be used (Brugnone et al., 1978; Veulemans et al., 1982; Perbellini et al., 1986). However, this measurement is invasive, and the difficulty in measurement is complicated by the fact that n-hexane is highly volatile. On the other hand, the measurement of unchanged n-hexane in alveolar air was recommended by the ACGIH in the past as a confirmatory test of exposure to n-hexane. This measurement could be useful for biological monitoring purposes because of its simplicity and high specificity and also because it is related to the concentration of n-hexane in blood. However, few studies have examined this indicator

(Filser et al., 1987; Brugnone et al., 1991; Periago et al., 1993; Cardona et al., 1996; Hamelin et al., 2004) and they couldn't reach a conclusion since the used methodology was different in the referred studies.

Hamelin and collaborators (2004) characterized, for 5 consecutive days, the relationship between n-hexane exposure (25 ppm and 50 ppm) and n-hexane excretion in alveolar air, and urinary 2,5-HD excretion in human volunteers. Through their study they conclude that both free urinary 2,5-HD and n-hexane in alveolar air measurements could be used for the biological monitoring of exposure to n-hexane. These authors consider that between these two indicators, n-hexane in alveolar air is less variable than 2,5-HD in urine, nevertheless the sampling time is more critical. After doing the study they suggest that biological monitoring of n-hexane based on the measurement of free urinary 2,5-HD is preferable to n-hexane in alveolar air.

1.3.2.3 Biomarkers of Susceptibility

Both the host and external environment factors affect many toxicant-induced health impairments and diseases and the metabolic enzyme gene polymorphisms on individual susceptibility to n-hexane-induced peripheral nerve damage, may be associated with a possible marker of susceptibility.

The differences in individual expressions of toxicity have become a hot point in toxicology because, although epidemiological studies have shown that occupational exposure is a major risk factor for n-hexane neuropathy, only a small fraction of workers exposed to n-hexane develop neuropathy (Zhang et al., 2006).

A marker of susceptibility could be a genetic factor such as the variants of metabolic genes for n-hexane that are involved in the procession of n-hexane neuropathy, as CYP2E1, and consequently individual susceptibility to peripheral nerve damage (Chang et al., 1993; Jenner, 1998). Metabolic interferences arising from habits and genetic polymorphisms should be carefully considered in order to explain inter- and intra-individual variability, even as biological conditions as sex, age, fatty mass, diseases and chronobiological factors (Mutti, 2001). Inter-individual differences in absorption, bioavailability, excretion and DNA repair should be taken

into account. Moreover, intra-individual differences, as a consequence of particular physiopathological alterations occurring in a specific period of time, also should be considered (Gil and Pla, 2001).

The CYP2E1 enzyme is responsible for metabolism of various aliphatic and aromatic hydrocarbons, solvents, and industrial monomers, including n-hexane and acetone. This isoenzyme is associated with free radical production and formation of endogenous toxins. Selectively localized in nigral dopamine-containing cells, CYP2E1 metabolizes n-hexane leading to the formation of its neurotoxic metabolite 2,5-HD (Jenner, 1998).

Iba and col. (2000) demonstrated that 2,5-HD formation following n-hexane exposure occurs more extensively in wild-type mice that in their CYPE21-deficient counterparts, indicating a requirement for CYP2E1 for 2,5-HD formation in the mouse. They also indicate that detoxification of metabolite may be more extensive in this species than in rat. The results suggest that overall formation of the γ-diketone may be higher in the rat than in the mouse.
According with Zhang and coworkers (2006), also polymorphism of CYP1A1 and GST genes are involved in the formation of 2,5-HD from n-hexane as well as in the elimination of 2,5-HD-formed electrophile.
In addition, differences in the development and maturity of phase I and phase II metabolic enzymes (specifically CYP2E1) between adults and children have been shown in several studies (Johnsrud et al., 2003; Ginsberg et al., 2002). Taken together, these data suggest that differences in metabolism of n-hexane may exist within the human population and between adults and children (Howd et al., 1983; US EPA/635/R-03/012).

Several authors concluded that CYP2E1 is highly polymorphic in the general population, could possibly lead to interindividual differences in the toxicity of chemicals metabolized. The polymorphism of this enzyme may be a susceptibility gene to n-hexane-induced peripheral nerve system dysfunction (Iba et al., 2000; Bolt et al., 2003; Zhang et al., 2006).

1.3.2.4 Biomarkers of Effect

Considering that the mechanism of toxicity of neurotoxicants is often very complex several biomarkers of neurotoxicity and of oxidative stress are used alone or in combination to evaluate their neurotoxic effects.

Biomarkers of neurotoxicity

The complexity of the nervous system and its distinctive peculiarities, together with the problems associated with determination of precise targets for neurotoxic action, does not allow a rapid and easy development of sensitive, specific and reliable biomarkers for neurotoxicity. Neurochemical measurements for detecting neurotoxicity in humans are limited by the inaccessibility of target tissue. The search for neurochemical parameters in peripheral tissues that are obtained easily and ethically in humans is the necessary approach for identifying and characterizing neurotoxicity, and could represent a marker for the same parameters in nerve tissue (Costa and Manzo, 1995).

As example we could refer the MAO-B activity. It is used clinically as a marker of pharmacological effects of MAO inhibitors, such as in the treatment of Parkinson's disease. The MAO-B activity in platelets has been used as a biomarker of the effects of styrene and perchloroethylene occupational exposures, which are known to cause dopamine depletion. Changes in MAO-B could represent an adaptive response to dopamine depletion and, alternatively, styrene or its metabolite(s) might exert a direct inhibitory effect on the enzyme (Gil and Pla, 2001).

Biomarkers of neurotoxicity were recently investigated by our research team, and Santos et al., (2012, 2013) selected AChE as a target of Mn in the CNS that may trigger or contribute to the development of oxidative stress suggesting that AChE activity may be an early biomarker of Mn neurotoxicity and risk assessment in populations exposed to this metal.
Neurotoxic metals were also studied by Andrade et al., (2013) who performed an *in vivo* study and tested delta-ALA-U and other biomarkers, as exposure and/or neurotoxicity biomarker in response to Pb, As or Mn treatments, as well as to a mixture of these 3 metals and they suggested

that delta-ALA-U levels may be used as a sensitive peripheral biomarker of exposure and neurotoxicity upon exposure to a mixture of Pb, As and Mn.

Biomarkers of oxidative stress

Industrial solvents such as n-hexane are capable of causing oxidative damage, especially through free radicals. In response to oxidative stress, there may be adaptive responses of the antioxidant systems, modification of cellular macromolecules and tissue damage.

Changes in the antioxidant systems and modified macromolecules can serve as biomarkers for a variety of xenobiotics. The protective systems include oxidized glutathione/reduced glutathione, glutathione reductase, catalase, superoxide dismutase and peroxidase activities. Macromolecules that may be affected by free-radical damage include lipids, proteins and nucleic acids (Gil and Pla, 2001).

With respect to the lipids, lipid peroxidation is started by the capture of a hydrogen from a fatty acid by an oxygen radicalar followed by the formation of hydroperoxides. The degradation of these hydroperoxides results in a series of derivatives with various compounds including carbonyl groups (Yin and Chen, 2005). The main compounds resulting from lipid peroxidation are aldehydes α,β-unsaturated as malondialdehyde (MDA), acrolein and HNE (4-Hydroxy-2 (E)-nonenal, and γ-ketoaldehydes as Isoketals (IsoKs) and Neuroketals (Picklo et al., 2002; Davies et al., 2004).

Lipid peroxidation of arachidonic acid mediated by free radicals, gives rise to H_2-Isoprostanes (H_2-IsoPs isomers of Prostaglandin H_2), which in turn lead to the formation of highly reactive γ-ketoaldehyde called "Isoketals" (IsoKs). The H_2-Isoprostanes can still suffer a non-enzymatic rearrangement giving rise to the F_2-Isoprostanes (F_2-IsoPs).
The quantification of the levels of F_2-IsoPs in plasma and urine has been used in studies of biomarkers of oxidative stress (Davies et al., 2007). According Sonnen et al., (2007) F_2-Isoprostanes have received considerable attention due to its chemical stability and minimal metabolism in situ, making them candidate biomarkers of lipid peroxidation.

The γ-ketoaldehydes are highly reactive and form covalent adducts with lysine residues and cross-linking with proteins at rates greater than HNE speed. Form Schiff bases adducts lactams and stable yielding hidrolactams (Picklo et al., 2002).

Studies have suggested the chemical basis for the extreme reactivity of IsoKs. The products of lipid peroxidation, such as HNE, acrolein and other carbonyls α,β-unsaturated, primarily react with proteins *via* Michael addition. Subsequently, these aldehydes generally adduct with Cys and to a lesser extent with lysine or histidine residues of proteins (Davies et al., 2004). The carbonyls α,β-unsaturated react with lysine to form the adduct hemiaminal, then dehydrate to form an imine. Both reactions are highly reversible, so we are in equilibrium in bulk form carbonyl. In contrast to the α,β-unsaturated, the γ-dicarbonyls, such as 2,5-HD, react rapidly to form the adduct hemiaminal (Fig. 1.6). The carbonyl group of hemiaminal can suffer intramolecular nucleophilic attack by the amine yielding a highly unstable pyrrolidine adduct.
A methyl 2,5-HD substitution increases the rate of formation of the pyrrole-adduct 10 times, probably through dialkyl effect. As IsoK is similar to dimethyl-2,5-hexanedione in relation to dialkyl substitution, is expected a rapid formation of pyrroles to IsoKs (Fig. 1.7) (Roberts et al., 1999; Davies et al., 2004, 2007).

Another important aspect concerning the alkyl substitution for γ-dicarbonyls is the impact of the oxidability of pyrrole adducts. The increased electron density of pyrroles formed from 3,4-dimethyl-2,5-hexanedione compared with those formed by 2,5-HD enables rapid oxidation in the presence of molecular oxygen. The oxidation of pyrrole leads to further cross-linking probably via formation of an electrophile which undergoes nucleophilic attack. The ability of γ-dicarbonyls substituted undergo cross-linking with proteins appears to be directly related to their susceptibility to oxidation.
The pyrrolidine then undergoes rapid dehydration to form the pyrrole adduct. These fast reactions are essentially irreversible, and quickly originate free γ-dicarbonyls pyrrole adducts.

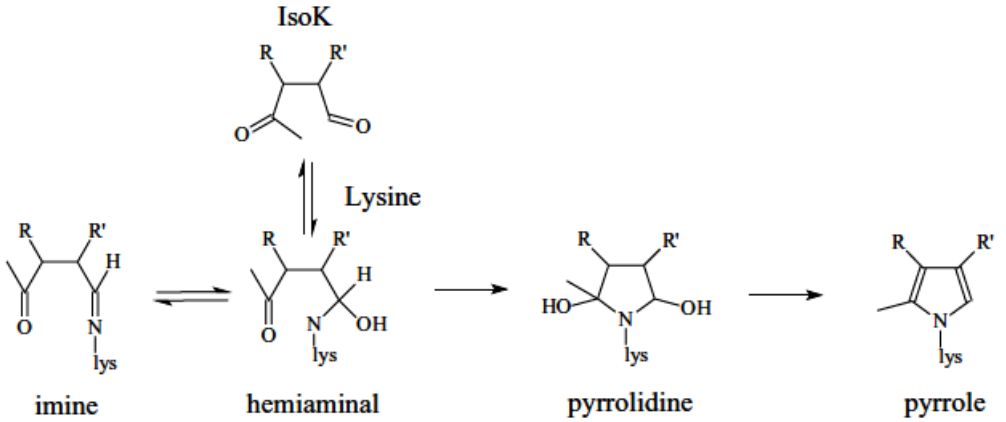


Figure 1.6 Proposed mechanism for imine and pyrrole adduct formation from isoketal reaction with lysine (Adapted from: Davies et al., 2004).

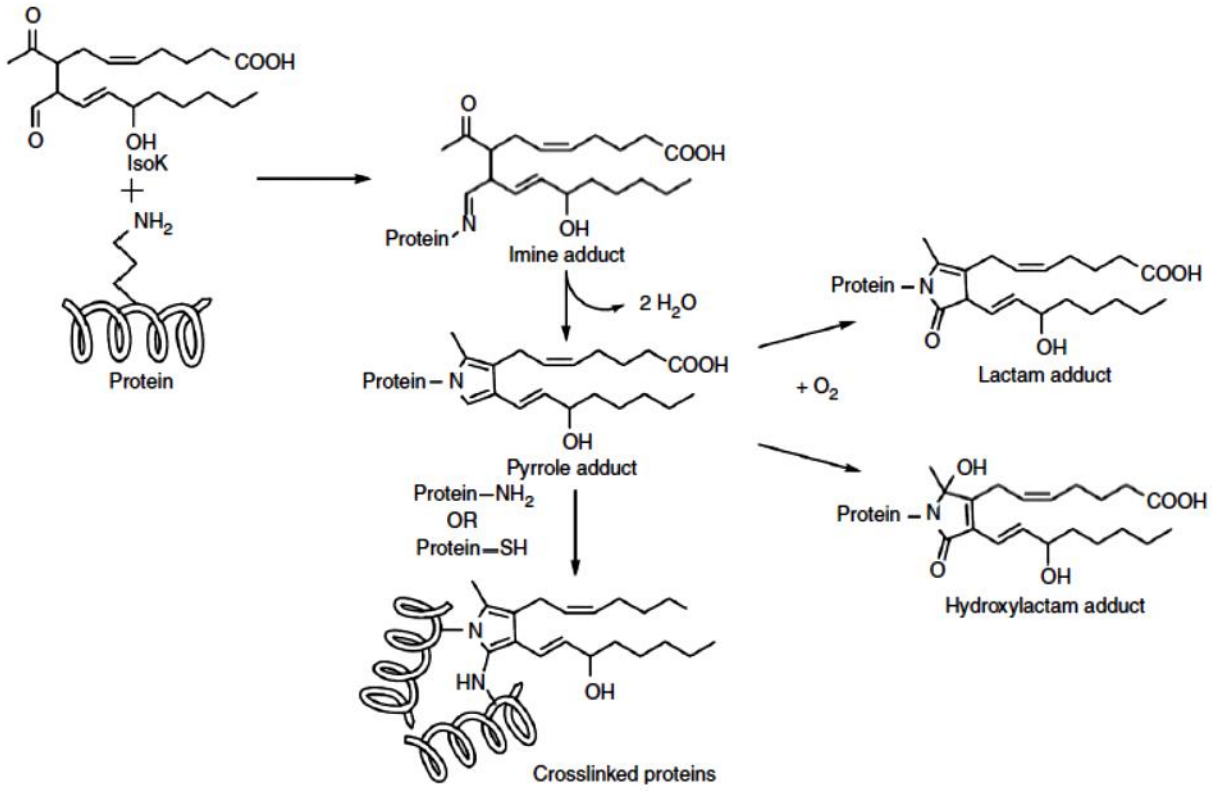


Figure 1.7 IsoK reacts with lysyl residues of proteins to form imine, pyrrole, lactam, hydroxylactam and crosslinked adducts (Adapted from: Davies et al., 2007).

The pyrroles formed by IsoKs should be more easily oxidized than those formed from dimethyl-2,5-hexanedione, due to the presence of an additional double bond in the pyrrole ring that may undergo conjugation (Davies et al., 2004, 2007).

Biomarkers of 2,5-HD neurotoxic effects

There isn't any validated biomarker of 2,5-HD neurotoxicity, however several studies have investigated endpoints of 2,5-HD toxicity to be selected as biomarkers of effect.

Experiments in rats exposed to γ-diketones have shown that formation of 2,5-HD-hemoglobin adducts is proportional to both: time and dose (Anthony et al., 1988; Costa, 1996). As the formation of adducts has been shown to be causally related to the development of neuropathy, hemoglobin may be seen as a surrogate for neurofilaments during *in vivo* exposure. As such, measurements of hemoglobin pyrrole adducts in the case of n-hexane are more than a biomarker of exposure and can be seen as a biomarker of effect. In this regard, more detailed dose-response studies in animals, as well as studies in humans, may be useful to arrive at a better quantitative assessment of neurotoxic risk linked to exposure to n-hexane or other γ-diketone precursors. As the cross-linking of neurofilaments is essential for neurofilament-filled axonal swellings (Graham et al., 1995), the ability to measure protein cross-linking in an accessible tissue would provide an additional means of assessing exposure to and health effects of n-hexane.

A protein present in erythrocytes, spectrin, has proved to be useful in this regard. The α and β subunits of spectrin are closely associated on the cytoplasmic side of the red blood cell membrane. Cross-linking compounds yield α,β-heterodimers that can be identified (in SDS polyacrylamide gels). Neurotoxic γ-diketones have been indeed shown to cause spectrin dimerization, while administration of nontoxic diketones does not cause spectrin cross-linking (Anthony et al., 1983; Genter et al., 1987, 1988; Costa, 1996). Erythrocytes are a source of stable proteins, and cross-linked spectrin can be detected in rats receiving daily ip injections of 2,5-HD.

Based on the mechanism of toxic action of 2,5-HD, some authors have proposed (Graham et al., 1995; Costa, 1996) the determination of adducts formed between pyrrole 2,5-HD and endogenous proteins as biomarkers of cumulative exposure to n-hexane, which reflects the amount of free 2,5-HD available to react with the target proteins. Thus, assessment of pyrrole

derivatives in serum and in urine (Mateus et al., 2002; Yin et al., 2014) may reflect the formation of pyrrole adducts, and be potential predictive biomarkers for neurotoxic effects.
However, these derivatives can either be present in the body due to exposure to certain xenobiotics, or due to genetic factors that determine changes in the physiological processes that induce the development of certain diseases (Landrigan et al., 2005; Sonnen et al., 2007).

The pyrrolization of proteins was observed in various diseases such as diabetes, Parkinson's disease, Alzheimer's disease, atherosclerosis, kidney disease, senile macular degeneration (Hidalgo et al., 1998; Martínez-Cruz et al., 2002) being pyrrole adducts formed as consequence of lipid peroxidation (Manini et al., 1998; Hidalgo et al., 2001; Gu et al., 2003; Davies et al., 2004).
The detection and characterization of these pyrroles may well be an index of the stress exerted by the cell in the body (Hidalgo et al., 1998), leading to the knowledge of the origin and the pathogenesis of some diseases, including neurodegenerative, even as a preventive warning system in order to facilitate timely therapeutic intervention in certain diseases (Gu et al., 2003).

1.3.2.5 Analytical methods to determine biomarkers of exposure

Analytical methods for routine analysis of biomarkers of exposure are mainly based on chromatographic techniques, both gas chromatography (GC) and liquid chromatography (LC) coupled with different kinds of detectors. GC is used for the determination of unchanged organic solvents present in exhaled air, blood, and urine. Identification of polar metabolites by gas chromatography-mass spectrometry (GC-MS) is not difficult, if these products tend to be relatively volatile and thermally stable (Manini et al., 2004). According with Manini et al., (2004, 2006), although the application of LC-MS in biomarker research is relatively young, recent studies clearly showed the potential of this technique in the determination of traditional biomarkers of exposure, as well as in the metabolism studies aimed at investigating minor metabolic routes.
Polar metabolites are molecules suitable for determination by LC. Efficient chromatography of acidic metabolites is obtained by using phosphate buffers, whereas retention of conjugated metabolites was obtained by ion-pair reversed phase (RP) chromatography.

Limitations due to the scarce sensitivity and selectivity of UV detection have been overcome by proper sample preparation: extensive sample clean up limits interferences from the matrix. Sample pre-concentration, derivatization with UV-chromophores or fluorescent reagents have been proposed to improve the characteristics of routinary used LC methods.

Exposure to n-hexane in occupationally exposed workers has been mostly assessed by determination of urinary levels of the toxic metabolite 2,5-hexanedione (Saito et al., 1991), being the urinary excretion of 2,5-HD an established biomarker of exposure to n-hexane and a suitable predictor of neurotoxic effects (IPCS 122, 1991; Mutti et al., 1984; Kawai et al., 1991b). Since most n-hexane metabolites are excreted only after conjugation with glucuronic acid, their determination by GC or HPLC requires preliminary hydrolysis (enzymatic and/or acid) followed by liquid–liquid extraction (Perbellini et al., 1981b; Fedtke and Bolt, 1987a,b; Perbellini et al., 1990a; Kawai et al., 1990, 1991a).

In the Table 1.4 is indicated the analytical/instrumental techniques used to detect biomarker 2,5-HD as well as the detection of pyrrole compounds in urine of animals exposed to n-hexane or 2,5-HD.

Throughout our work we use as analytical techniques, the spectrophotometric method of Ehrlich, GC-MS and ESI-LC-MS/MS techniques.

Table 1.4 Analytical/instrumental techniques used to detect biomarker 2,5-HD or pyrrole compounds in urine of animals exposed to n-hexane or 2,5-HD.

Parent compound	Biomarker	Method	Sample preparation	Internal standard	Reference
n-Hexane	2,5-HD	GC-FID	Yes	Yes	Saito et al., 1991
n-Hexane	2,5-HD	LC-APCI-MS	Direct injection	None	Andreoli et al., 1998
n-Hexane	2,5-HD	GC-MS ESI-LC/MS(MS)	Yes Direct injection	Yes None	Manini et al., 1998
n-Hexane	PLS	UV-Vis Spectrom. Ehrlich Method	Yes	None	Mattocks and White, 1970
2,5-HD	PE	UV-Vis Spectrom. Ehrlich Method	Yes	None	Mateus et al., 2000
2,5-HD	DMPN	LC-MS/MS	Direct injection	None	Torres et al., 2014

PLS: Pyrrole-like substances; PE: Pyrrole equivalents; DMPN: dimethylpyrrolenorleucine

1.4 MECHANISM OF TOXICITY OF PYRROLE COMPOUNDS

1.4.1 DIMETHYLPYRROLE ADDUCTS

Several studies showed that 2,5-HD could react directly with axonal protein by the formation of N-substituted 2,5-dimethylpyrrole adducts at ε-amine nitrogen of the lysine residues of neurofilaments (Fig. 1.8) (Pyle et al., 1992; Graham et al., 1995; DeCaprio et al., 1997).
Secondary oxidation of the pyrrole ring to an electrophile reacted with neurofilament nucleophiles resulting in intra- and intermolecular protein cross-linking, and this was considered to be the determinant event in 2,5-HD neuropathy (Graham et al., 1982; DeCaprio, 1986; Sayre et al., 1986; Genter et al., 1988; DeCaprio et al., 1988; Zhu et al., 1994).

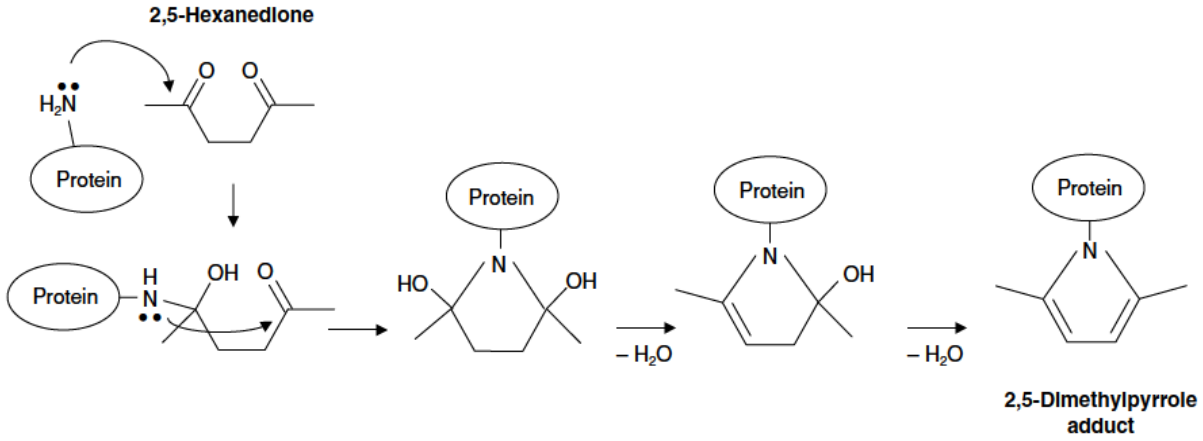


Figure 1.8 Sequential covalent binding of the electrophile 2,5-hexanedione to lysine residues of axonal proteins results in the formation of a 2,5-dimethylpyrrole adduct. (Adapted from: Boelsterli, Urs A., *Mechanistic toxicology: the molecular basis of how chemicals disrupt biological targets*. 1st ed., Taylor & Francis, London, 2003).

According to LoPachin and DeCaprio, (2004) pyrrole adduct formation in NF proteins has been proposed to result in physicochemical changes in the proteins sufficient to interfere with the ability of mobile NF proteins to interact with the polymeric cytoskeleton and to cause NF accumulation and nerve degeneration (Fig. 1.2). As a result, the adducted subunit remains attached to the kinesin transport vector which, in the absence of compensatory changes in gene expression or synthesis, promotes a loss of NF protein content per unit area of axon.

Pyrrolylated proteins are susceptible to attack by molecular oxygen and most of alkylpyrroles undergo the same reaction (DeCaprio and O'Neill, 1985).
It was observed that autoxidation of alkylpyrrole adducts *in vivo* (DeCaprio 1986; Lapadula et al., 1986; Carden et al., 1986) and in vitro (DeCaprio et al., 1982; Zhu et al., 1993) leads to the cross-linking of numerous proteins, including NF proteins. Pyrrole oxidation-mediated covalent cross-linking of NF proteins has been proposed as the second obligatory step in γ-diketone neuropathy (Graham et al., 1982; Genter et al., 1988; Zhu et al., 1995).

Zhu and coworkers (1993, 1994) showed that 2,5-dimethyl-N-alkylpyrroles are chemically reactive in aqueous solutions at physiologic temperature and undergo spontaneous and rapid autoxidation to form pyrrole dimers and trimmers (Fig. 1.9 B). A clear understanding of the

reaction of autoxidation of N-substituted 2,5-dimethylpyrroles is a prerequisite for the elucidation of a role for protein cross-linking in 2,5-HD neurotoxicity.

The oligomers compounds obtained from autoxidation reaction of 2,5-dimethyl-N-alkylpyrroles contain methylene bridges between C-2 of one pyrrole ring and C-3 of a second ring (Zhu et al., 1994, 1995). The same autoxidative dimerization occurred when NAL or lysine containing dipeptides are incubated with 2,5-HD.
The secondary autoxidative phenomena following gamma-diketone-treated protein that originates pyrrole adduct formation proceed free radical-mediated mechanisms (DeCaprio, 1986).

Thus, pyrrole-to-pyrrole linkages mediate by 2,5-HD-derived protein cross-linking and autoxidative pyrrole-pyrrole dimerization may involve free radical chain reaction (Zhu et al., 1993, 1994, 1995). In addition to autoxidative dimerization of pyrrole compound, alternative cross-linking reactions may involve oxidized pyrrole rings and protein nucleophiles, such as thiols and amines (Fig. 1.9 A and C) (Tshala-Katumbay, 2005, 2008, 2009a).

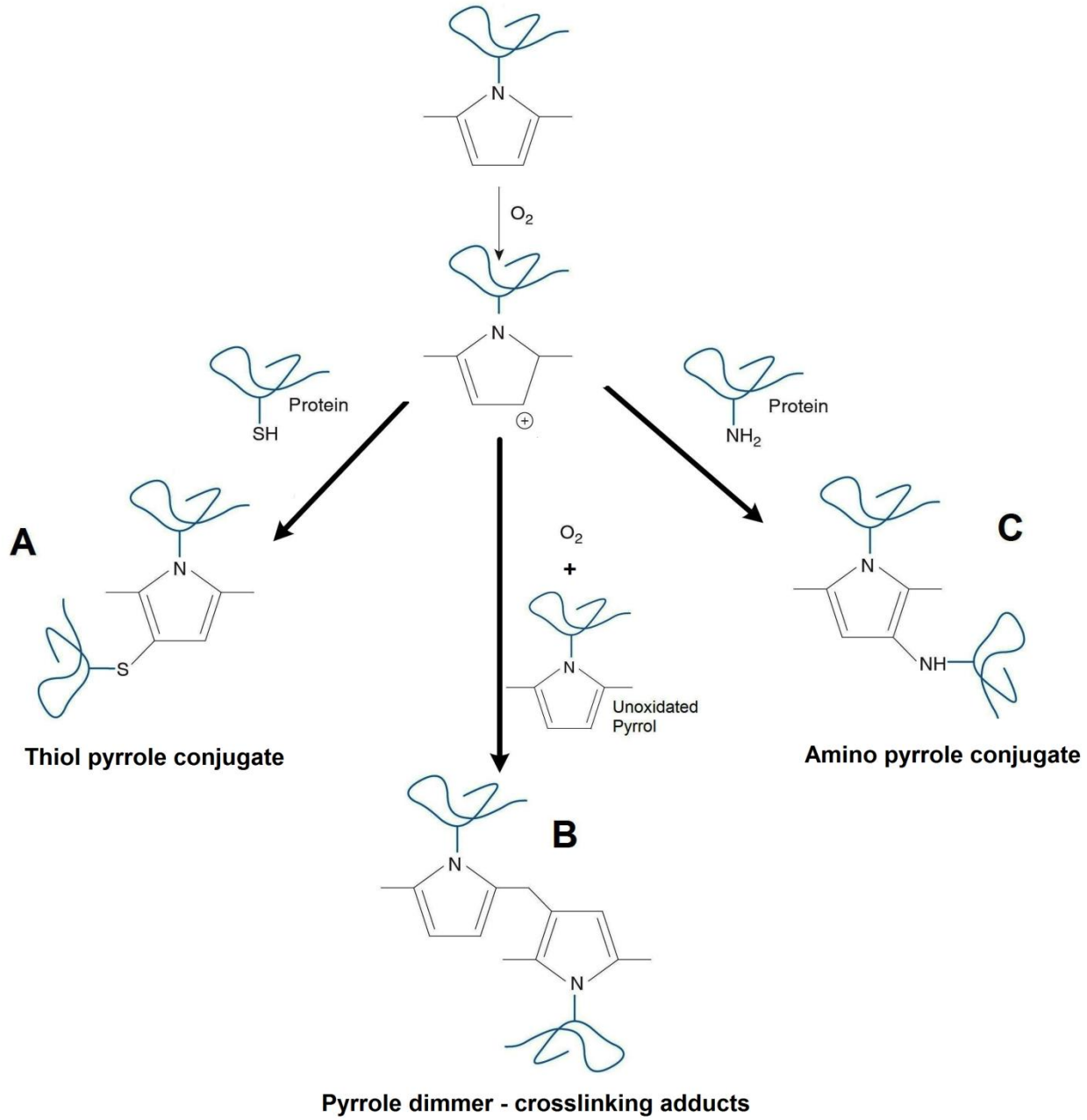


Figure 1.9 Autoxidative dimerization of pyrrole compound (B) and alternative cross-linking reactions involving pyrrole rings and protein nucleophiles, such as thiols (A) and amines (C). (Adapted from Moser et al., In *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 8th ed. McGraw-Hill, New York, 2013, and Mateus M.L.; *Avaliação e Prevenção do Risco de Exposição a Misturas com n-Hexano: Parâmetros Bioquímicos e Comportamentais*. Faculdade de Farmácia, Universidade de Lisboa, Lisboa, 2002).

1.4.2 PYRROLE COMPOUNDS AND FREE RADICALS

Pyrrole dimerization may involve free radical chain reactions, and biological nucleophiles namely NAC and reduced glutathione (GSH) could directly or indirectly affect the formation of pyrrole dimers by inhibiting this process (Amarnath, et al., 1994; Zhu et al., 1995).

Free radicals promote oxidation, because contain one or more unpaired electrons, act as electron acceptors and essentially "steal" electrons from other molecules. They are referred to as oxidizing agents because they tend to induce other molecules to donate their electrons.

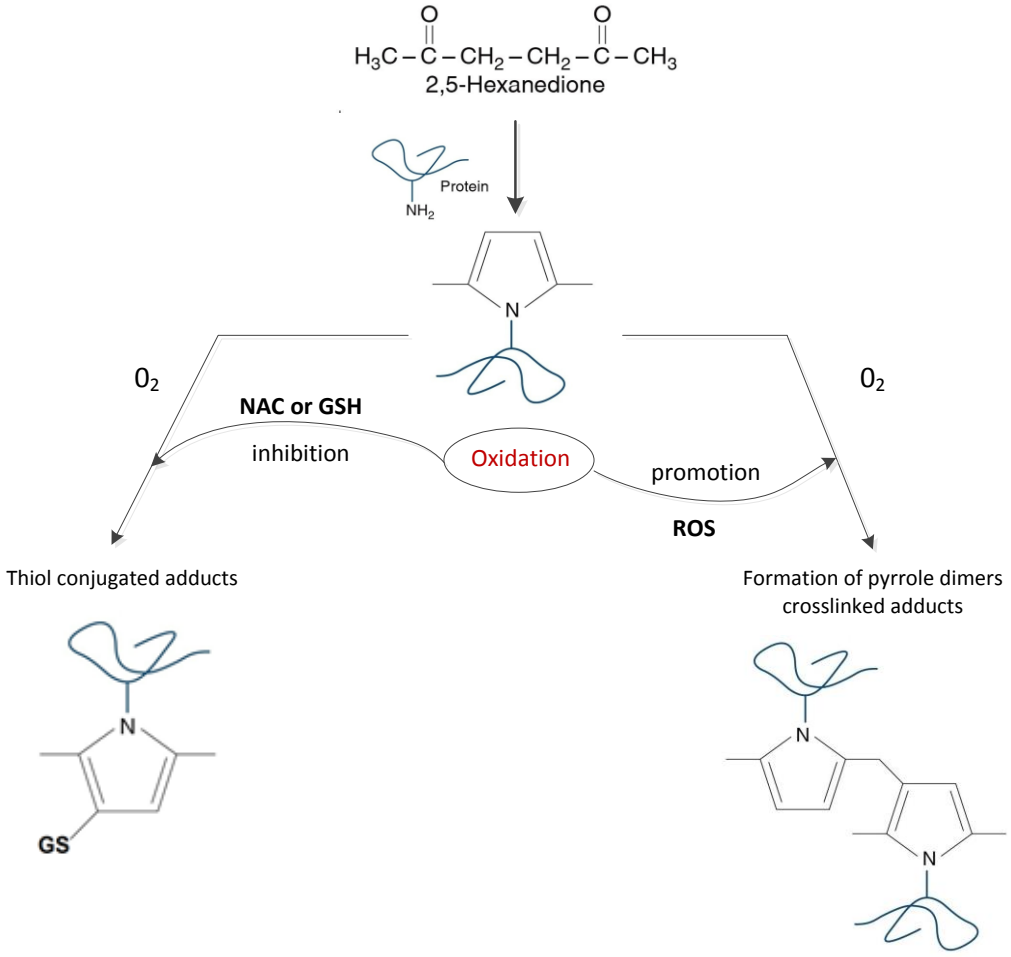


Figure 1.10 Proposed mechanism of formation of thiol conjugated adducts, *via* Thiol-to-Pyrrole conjugation and direct antioxidation action, and formation of pyrrole dimers, *via* autoxidative Pyrrole-to-Pyrrole Cross-linking (Adapted from Zhu et al., 1995).

We are constantly exposed to free radicals created by electromagnetic radiation from the environment, both natural (*e.g.*, radon, cosmic radiation) and man-made, and by internal cellular

metabolism. The most common cellular free radicals are hydroxyl radical (OH●), superoxide radical (O2-●), and nitric oxide (NO●). Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrate (ONOO), are not free radicals, nevertheless can lead to their generation through various chemical reactions. Free radicals and related molecules are often classified together as reactive oxygen species (ROS) to signify their ability to promote oxidative changes and oxidative stress within the cell (Gilgun-Sherki, 2001).

The presence of free radicals promotes the formation of crosslinked adducts (Amarnath, et al., 1994; Zhu et al., 1995). In fact secondary autoxidative reactions of the formed alkylpyrroles leads to electrophilic pyrrolylmethyl intermediates followed by their capture by unoxidized second pyrroles, forming pyrrole dimers which produce intra and intermolecular cross-linking in neurofilaments (NF) proteins. If, as has been proposed, covalent cross-linking of NF proteins is critical to the mechanism, it is conceivable that biological thiols could exert protective effects against γ-diketone neuropathy by interrupting the cross-linking process (Fig. 1.10). Alternatively, a lack of effect of GSH would be consistent with the hypothesis that pyrrole formation itself is sufficient to cause neuropathy.

1.4.3 ENDOGENOUS PRODUCTION OF PYRROLE COMPOUNDS

The presence of 2,5-HD in subjects who have not been occupationally exposed to n-hexane (or methyl-*n*-butyl ketone) is referred by several authors and is important to determine its origin in the human body (Brugnone et al., 1991; Mutti et al., 1993; Bavazzano et al., 1998).

Canesi et al. (2003) found in urine samples of persons not exposed to n-hexane 372±47 μg/mL of 2,5-HD, and Kessler et al., (1990), reported that 2,5-HD originates pyrrole substances that were identified and defined as PLS "pyrrole-like substances". In addition, pyrrolic compounds not originated from 2,5-hexanedione, *e.g*. urobilinogen, are normal constituents of urine and can be detected by Ehrlich's reagent.

In people not exposed to n-hexane, 2,5-HD and 4,5-dihydroxy-2-hexanone are products mainly deriving from intermediate metabolism in the human body. Only a minimal part may derive from

n-hexane as a ubiquitious micropollutant. The presence of n-hexane and its isomers in gasoline and fuels (6%) makes this solvent a ubiquitous pollutant for which reference biological levels in the general population need to be defined. Such trace amounts might originate from atmospheric contamination or endogenous production of hexane, *e.g.* by lipid peroxidation (Perbellini et al., 1993; Manini et al., 1998). According with Brugnone et al., (1991), despite the possibility of this endogenous production of aliphatic short-chain hydrocarbons, seems reasonable they conclude that urinary 2,5-HD found in the general population should be considered to have derived from inhaled n-hexane rather than from an endogenous production.

1.5 PYRROLE COMPOUNDS AND NEURODEGENERATIVE DISEASES

Epidemiological studies and basic molecular research have led to the supposition that exposure to chemicals in the environment contributes to the onset and development of human neurodegenerative diseases (Eisen, 1995; Landrigan et al., 2005; LoPachin et al., 2008a,b). These diseases result from the gradual and progressive loss of neural cells, leading to nervous system dysfunction. Known risk factors for neurodegenerative disease include certain genetic polymorphisms and increasing age. Other possible causes may include gender, poor education, endocrine conditions, oxidative stress, inflammation, stroke, hypertension, diabetes, smoking, head trauma, depression, infection, tumors, vitamin deficiencies, immune and metabolic conditions, and chemical exposure (Brown et al., 2005).

A number of studies have examined the relationship between exposure to environmental agents and neurologic outcomes, since exposure to numerous structurally diverse chemicals can cause significant morphological and functional damage to nerve cells in the CNS and PNS (Brown et al., 2005; LoPachin and DeCaprio, 2005). Table 1.5 summarizes the clinical features of some of the major neurodegenerative diseases (Singh et al., 2004).

Because the pathogenesis of many of these diseases remains unknown, we must consider the role of environmental factors in these diseases.

Despite significant research, the pathophysiological and molecular mechanisms for many neurotoxicants remains poorly defined. However, a common mechanistic theme in the toxicological sciences has been the formation of chemical adducts with macromolecules.
Presumably cell toxicity occurs when adduct formation disrupts the structure and/or function of macromolecules (Fig. 1.2) (Nelson and Pearson, 1990; Hinson and Roberts, 1992). Since many neurotoxicants and their metabolites are electrophiles, it is possible that adduct formation is also a critical step in the neuropathogenic processes initiated by exposure to these chemicals (LoPachin and DeCaprio, 2005; LoPachin et al., 2008).

Table 1.5 Characteristics feature of a few neurodegenerative (NDG) diseases.

NDG diseases	Clinical features	Neuropathology
Alzheimer's Disease (AD)	Dementia, progressive deterioration of thought, judgment, language skills, visual-spatial perception and mood	Generalized cortical atrophy with shrinkage of the amygdala and hippocampus. Selective dysfunction and death of neocortex, hippocampus, amygdala, basal forebrain, and brainstem. The senile plaque, an extracellular deposit of amyloid, composed of Ab peptide derived from amyloid precursor protein.
Amyotrophic Lateral Sclerosis (ALS)	Progressive weakness and atrophy of skeletal muscles. Weakness of chest muscles and diaphragm and muscle dysfunction in the larynx and pharynx lead to respiratory problems and death with bronchopneumonia.	End stage disease is characterized by loss of primary motor neurons in the neocortex. Motor neurons have abnormal phosphorylated neurofilaments.
Parkinson's Disease (PD)	Slowness of voluntary movement (bradykinesia), rigidity, and tremor. Cognitive deficits (dementia), *i.e.*, post encephalitic parkinsonism. A mitochondrial toxin called MPTP induces parkinsonism.	Neuronal degeneration due to the loss of the pigmented neurons in the substantia nigra (pars compacta). The toxic metabolite MPP^+ derived from MPTP, inhibits the mitochondrial complex I leading to ATP depletion and generation of toxic oxygen free radicals.

(Adapted from: Singh et al., 2005).

The protein adduct formation could result in toxic neuropathies as adduct formation could negatively impact the tertiary structure and/or function of these proteins and thereby interfere with, for example, energy metabolism, axonal transport or presynaptic neurotransmitter release. It is also possible, for electrophilic neurotoxicants to produce specific effects by reacting with functionally critical nucleophilic centers on proteins that regulate cellular pathways or processes. Understanding the chemistry and pathophysiological consequences of adduct formation could not only benefit neurotoxicology, as yet might also provide insight into mechanisms of human neurodegenerative diseases.

The compounds originated by the reactions resulting from the action of the reactive oxygen species are also associated with various neurodegenerative diseases.
For Alzheimer's disease (AD), high levels of HNE were found in brains and cerebrospinal fluid (CSF) of AD patients. Also in cerebrospinal fluid products resulting from the action of free radicals in carbohydrates, was found. It was also found high levels of acrolein in the amygdala area. Also in hippocampus and inferior parietal lobule, products generated by oxidation by hydroxyl radical, the amino acid side chains or derived by the alkylation of multifunctional products such as acrolein and HNE were found (Picklo et al., 2002).

1.5.1 ALZHEIMER'S DISEASE

Alzheimer's disease represents the most common form of dementia in the elderly, characterized by progressive loss of memory and cognitive capacity severe enough to interfere with daily functioning and the quality of life. The disease is characterized by extracellular amyloid deposits or plaques and by the presence of intraneuronal neurofibrillary tangles composed of aggregates of hyperphosphorylated tau protein (Migliore and Coppedè, 2009).
The neurodegeneration associated with Alzheimer's disease (AD) appears to involve oxidative damage characterized by protein oxidation (Hensley et al., 1995; LoPachin and DeCaprio, 2005), increased expression of antioxidant enzymes (Pappolla et al., 1998), and elevated lipid peroxidation (Sayre et al., 1997). Evidence suggests that the AD process involves several factors (genetic, age-related, and environmental) that converge to initiate neuronal oxidative stress and a subsequent pathophysiological cascade. Lipid peroxidation, a major consequence of oxidative

stress, is the free-radical driven fragmentation of polyunsaturated fatty acids and is a biochemical hallmark of AD (Arlt et al., 2002; Keller and Mattson, 1998; Montine et al., 2002; Zarkovic, 2003; LoPachin et al., 2008).

As we have referred, lipid peroxidation produces several α,β-unsaturated aldehydes (*e.g.*, acrolein, malondialdehyde, 4-hydroxy-2-nonenal) among which, acrolein has the highest nucleophilic reactivity (Picklo et al., 2002; LoPachin and DeCaprio, 2005). Acrolein-protein adducts have been detected in brains of AD patients, and are considered to be a biomarker of the accompanying oxidative stress (Uchida et al., 1998). In addition, it has been hypothesized that acrolein adduct formation with lysine residues on tau proteins might play a role in the development of neurofibrillary tangles that are a pathological hallmark of AD (Calingasan et al., 1999; Picklo et al., 2002).
It is interesting to note that acrolein is a ubiquitous environmental pollutant (LoPachin and DeCaprio, 2005). Thus, the pathophysiology of AD could involve protein adduct formation mediated by both endogenous and exogenous neurotoxic components.

The contribution of adducts formed between IsoKs and proteins to cellular dysfunction also remains an area of considerable interest because initial findings suggested that these adducts contribute to the mechanism of Alzheimer's disease. Exogenous IsoKs inhibit the mitosis and microtubule aggregation, break the blood-brain barrier and induce necrosis in tissues of the brain (Davies et al., 2007).

1.5.2 PARKINSON'S DISEASE

The growing scientific consensus is that Parkinson's Disease (PD) is not a single disorder, however instead reflects a common pathological endpoint resulting from the interaction of various environmental and genetic risk factors. Thus, the risk attributable to any single factor is likely to be small and to differ depending on specific population characteristics.
Although genomic screening studies are steadily producing more data relating genes to PD (Martin et al., 2001; Scott et al., 2001; Moore et al., 2005), there is evidence that also environmental factors are involved (Tanner et al., 1999), and the generally accepted hypothesis is that the disease is the result of an interaction between the two components.

It has been suggested that individuals with particular genotypes may have difficulty in metabolizing one or more environmental neurotoxicants and that this "poor metabolizer" status could make them susceptible to developing PD following exposure to such neurotoxins (Pezzoli et al., 1995; Canesi et al., 2003).
Toxin accumulation could produce selective neurodegeneration in the substantia nigra through mechanisms involving oxidative stress (Moore et al., 2005).
Hydrocarbon-solvents are environmental toxins that appear to be involved in the pathogenesis of PD. Prolonged exposure to these substances has been associated with PD in a number of case reports (Gralewicz and Dyzma, 2005).

Moreover, a large study in PD patients has recently shown that chronic exposure to hydrocarbon-solvents contributes towards earlier onset of PD and more severe disease throughout its course (Pezzoli et al., 2000; Canesi et al., 2003). One of the compounds involved was n-hexane, which is a neurotoxin that can induce a very well described peripheral neuropathy and also cause damage to the central nervous system (IPCS 122, 1991).
As we already have referred, n-hexane and its derivatives are common environmental contaminants, and they are also present in the human body independently of environmental micropollution as by-products of lipid peroxidation (Fedtke and Bolt 1986a; Vaz and Coon, 1987).
Canesi et al., (2003) show that advancing age and PD are both associated with a reduction in 2,5-HD and 2,5-DMP urinary levels. The lower 2,5-HD blood and urinary levels could be due either to reduced conversion of n-hexane to 2,5-HD or to an increase in the catabolization of 2,5-HD. The finding of lower urinary levels of 2,5-DMP, which is a metabolite of 2,5-HD, rules out an increase in the catabolism of 2,5-HD, suggesting that the conversion of n-hexane to 2,5-HD is reduced in PD patients and elderly controls (Vanacore et al., 2000).
The reduction in the catabolisation of n-hexane was related to age and the incidence of PD increases with age (Canesi et al., 2003). The characteristic Lewy bodies found in the brain of patients with PD or Lewy Body Disease and in about 10% of elderly patients contain ubiquitin-positive protein conjugates. These are present also in the intermediate filament aggregates found in the peripheral and central nervous system of rats exposed to n-hexane (Savica et al., 2010). Thus, aging and PD may be associated with a reduction in the capacity to eliminate the

hydrocarbon n-hexane. This metabolic alteration may play a role in the pathogenesis of PD (Vanacore et al., 2000).

1.6 PROTECTION AGAINST n-HEXANE NEUROTOXICITY

Occupational exposure

The best way to avoid outbreaks is to use a cleaning solvent with very low n-hexane content, or better still, to find a non-toxic substitute for n-hexane. Short of this, safety regulations and guidelines in handling n-hexane must be strictly adhered to. Workers should be periodically screened for the development of toxicity (Chang et al., 1993).

Table 1.6 shows a list that is not exhaustive, rather gives an indication of the likely intensity of exposure in a range of occupations. The actual solvent exposure is determined by a number of factors including which solvent is used, in what concentration, the method of use, adequacy of ventilation, and the personal protective equipment employed. For example, a house painter applying water based paint by brush in a well-ventilated room will have considerably lower exposure than one spray painting high solvent paint in a poorly ventilated cellar.

Table 1.6 Occupation with exposure to solvents (including n-hexane).

Occupation	Intensity of solvent exposure
Dry cleaning, screen printing, rotogravure printing, industrial painting, manufacture of glass reinforced plastic, tile fixing	High exposure
House painting, mechanic, assembly processes using solvents, paint making, industrial degreaser	Moderate exposure
Petrol pump attendant, joiner/carpenter, chemical process operator, laboratory technician, cleaner using polishes	Low exposure

(Adapted from: Dick, 2006)

In the occupational health context, atmospheric levels should be kept below the recommended occupational exposure limits by suitably designed work processes and engineering controls

including ventilation. Suitable protective clothing and respiratory protection should be readily available for use in enclosed spaces, in emergencies, and for certain maintenance operations. The use of a supplied-air respirator or a self-contained breathing apparatus in continuous flow mode for n-hexane concentrations up to 8 800 mg/m^3 (2 500 ppm) is recommended. It should be fitted with a full face piece for concentrations between 8 800 mg/m^3 (2 500 ppm) and 17 600 mg/m^3 (5 000 ppm). Although, protective gloves may resist to penetration by n-hexane, mixtures of n-hexane with other solvents, such as methyl ethyl ketone, may permit the n-hexane to penetrate gloves and other protective clothing. This factor should be considered when using n-hexane mixtures (IPCS 122, 1991).

Environmental Exposure

At the levels of n-hexane to which the general population is exposed, is unlikely to be any hazard except in the case of major spills or discharges where there could be transient local effects (IPCS 122, 1991).

1.6.1 CHEMICAL PROTECTION

In a perspective of protection of human health, it is important to study protective agents and/or antagonists of n-hexane, which may interfere with the formation of 2,5-HD and/or of pyrrole adducts, due to environmental of occupational exposure as the occurrence of certain diseases.
In this context, risk prevention against n-hexane neurotoxicity is a relevant issue towards the measures to be proposed in occupational toxicology. One of the approaches that could be applied in risk protection against n-hexane neurotoxicity is the development of a 2,5-HD antagonist that could interfere with its chemical mechanism of toxicity.

1.6.1.1 Role of Zinc

Zinc is a transition element that has the ability to form coordinating bonds with electronegative atoms (oxygen, sulphur and nitrogen) and Zn (II) is an essential ion and is required as cofactor for many metalloenzymes. It regulates metallothionein (MT) synthesis and can interact with radical chemical toxicity of several compounds (Bertini et al., 1994).

Mateus and co-workers (2000, 2001, 2002), investigated the protective role of zinc against the neurotoxic effects of 2,5-HD using *in vivo* models, where two different routes of exposure (oral and ip) were tested rats exposed to 2,5-HD and rats co-exposure to 2,5-HD and zinc (2,5-HD+Zn). They found a decrease in the excretion of pyrroles derivatives and free 2,5-HD as well as a recovery in motor activity performance was found in rats co-exposed to (2,5-HD+Zn) (Mateus et al., 2000, 2001). The mechanism of this interaction was also investigated applying chemical models simulating the *in vivo* synthesis of dimethylpyrroles using the reaction of 2,5-HD with NAL in presence or absence of a zinc compound (Mateus et al., 2000). The prevailing mechanism of this interaction was also investigated in rats co-exposed and pre exposed to zinc acetate and it was concluded that on comparing the changes in pyrroles excretion, there was a similar decrease in urinary excretion of pyrroles in the two groups of rats exposed to the mixture as compared with the pyrroles excreted in rats exposed to 2,5-HD alone (Mateus et al., 2000, 2002). The results obtained suggest that zinc coordinates mainly to amino groups inhibiting the formation of 2,5-HD lysine complexes (Mateus et al., 2001). However, the simultaneous depletion of free 2,5-HD does not exclude the possibility of a simultaneous coordination of zinc ion to 2,5-HD (Mateus et al., 2000). It is also feasible that this process is affected by other factors, such as the reversibility of metal complexes formation and the binding of zinc ion to other endogenous critical proteins (as MT), which could add new approaches to evaluate the definitive role of this metal ion in the overall process.

More recently, it has also been reported that in certain degenerative diseases zinc may delay their progression (Gu et al., 2003; Jackson et al., 2000, 2010).

Pfeiffer et al., (1974) performed a study with outpatient schizophrenics and found they had low levels in serum zinc. They also found approximately 50 percent of the patients had low in blood histamine (histapenic) and high in copper, while 20 percent were high in blood histamine (histadelic) and normal in serum copper.

Zinc is needed by the mast cell in order to store histamine. The terminal vesicles of the mossy fibers of the hippocampus are high in zinc which may be needed to store histamine for histaminergic neurotransmission.

With specific therapy based on changing low or excess tissue histamine, most problem patients improved. A group of 30 percent to 40 percent of patients was normal in their blood histamine and normal in serum copper levels. This was the group which excreted kryptopyrrole (KP) or the

"mauve factor." They also found that these patients respond to large doses of B6 and dietary supplements of zinc because the pyrrole combines with pyridoxal (Vit. B6) and then makes a complex with zinc and produces a combined disease deficiency. Some patients had both, the mauve factor and a histamine imbalance. The schizophrenias may truly be a series of three or more biochemical imbalances.

According with Pfeifer et al., (1974) several authors have confirmed the original observations that abnormal mauve factor is excreted in greater frequency in the urine of schizophrenics. Recently, these authors have succeeded in isolating and identifying the mauve factor as 2,4-dimethyl-3-ethylpyrrole (isomers), an observation confirmed subsequently by Brett Lambert (Director of Applied Analytical Laboratories, Australia, in "Overview of Urinary Pyrrole/Mauve Factor Analysis", 2013).

The dose of Vit. B6 needed by the KP-positive patient may be as high as 3000 mg per day to prevent Psychopathology and keep the urine free of KP. They found that KP combines with the aldehyde of pyridoxal phosphate to form a stable Schiff's base which is then excreted in the urine complexed with zinc. This results in a syndrome of Vit. B6 and zinc deficiency. Mauve-positive patients excrete in their urine significantly more zinc and coproporphyrin.

In addition, they experimented in "schizophrenics" compared to "normals" an oral dose of 50 mg of Vit. B6 which result in the reduction of urinary zinc excretion. This 50 mg oral dose of Vit. B6 reduce urinary zinc excretion. Decrease in zinc excretion produced by the B6 is greater in schizophrenic patients, comparing the levels with "normal".

According with Jackson et al., (2010) patients with various types of physiological and psychological stress had high urine pyrrole levels. They reported that due to the chemical structure of the pyrrole molecule, certain patients excreting high levels of pyrroles also excrete excess amounts of zinc and vitamin B6 in their urine (Jackson et al., 2000).

Pfeiffer et al., (1974) report another study with a female. She showed a syndrome of severe constipation and splenic pain. She also had seizure syndrome and some mental problems. They found her KP excretion urinary were very high, and found further that abnormalities decreased with zinc and B6 therapy. They referred that a therapy with vitamin B6 taken with zinc keeps all mental and abdominal symptoms away.

1.6.1.2 Role of GSH and NAC

The central role of GSH

The brain is among the major organs generating large amounts of reactive oxygen species and is especially susceptible to oxidative stress. The biological thiol, glutathione (GSH), plays a critical role as antioxidant, in protecting cells from oxidative stress, and xenobiotics, as well as maintaining the thiol redox state, most notably in the central nervous system (CNS). GSH also plays a critical role as enzyme cofactor, Cys storage form (the major redox buffer), a neuromodulator in the CNS, participates in synthesis of leukotrienes and prostaglandins and GSH deficiency has been implicated in neurodegenerative diseases.

The tripeptide glutathione (GSH, γ-glutamyl-L-cysteinyl glycine) is nearly ubiquitous in cells and has been shown to participate in a multitude of cellular functions and many of these are associated with protection against reactive intermediates, such as, free radicals and electrophiles, which include metabolites resulting from activation or detoxification of exogenous and endogenous compounds. Thus, GSH contributes with protein thiols for the maintenance of a suitable thiol redox balance which is crucial for cellular homeostasis (De Flora et al., 2001; Townsend et al., 2003; Aoyama et al., 2008). Although GSH was seen for many years as a protective molecule against activated metabolites of xenobiotics and of normal oxidative products of cellular metabolism, it is now also known to play a role in bioactivation of certain molecules (*Current Protocols in Toxicology*, 1999).

GSH is predominantly located intracellularly and its biosynthesis occurs in the cytosol of all cells, however the liver plays a particular role in its synthesis. Its biosynthesis involves a two-step process, both of which are ATP dependent:

L-glutamate + L-Cysteine + ATP → L-γ-glumatyl-cysteine + ADP + Pi

γ-glumatyl cysteine synthetase

L-γ-glumatyl-cysteine + glycine + ATP → **GSH** + ADP + Pi

glutathione synthetase

γ-Glumatyl Cys synthetase (γ-GCS), and glutathione synthase are the two enzymes that catalyze the two reactions of biosynthesis of GSH. The rate-limiting step in GSH synthesis is the availability of Cys (10^{-4} M), because glutamine and glycine are present at higher concentrations

(10^{-3} M) (Griffith and Meister, 1979; Drigen, 2000; De Flora et al., 2001; Dickinson and Forman, 2002; Aoyama et al., 2008).

Most mammalian cells can synthesize GSH, however is mainly the liver that takes up and removes the bulk of the dietary Cys (in the portal blood), mostly by converting it to GSH, which then is released mainly into the blood circulation (Stipanuk et al., 2006). Although Cys be necessary in other tissues for GSH synthesis it returns to the liver, which closes the GSH cycle.

GSH is used for the functions described above and also can be exported. Export of GSH allows the cell to protect its membranes against oxidative or other forms of damage by keeping thiol groups and membrane components (*e.g.*, vitamin E) in their reduced forms (Meister, 1994).

GSH breakdown

When GSH breakdown, plasma GSH derived from the liver can only be utilized by tissues that have considerable amounts of γ-glutamyl transpeptidase (GGT) activity (*e.g.*, kidney, small intestine, and type 2 alveolar cells). Degradation of GSH begins at the cell surface and requires enzymes located both on the cell surface (GGT) as well as in the cytosol. GGT is located at kidney epithelial cells. The kidneys take up GSH from the plasma through breakdown of GSH via GGT activity. GSH, GSSG, and γ-glutamyl GSH react with GGT at the outer surface. Most plasma GSH, transported to the kidney, is degraded with a half-life of 1.5 sec (Dickinson and Forman, 2002; Forman et al., 2009).

This salvage pathway for glutathione was proven through inhibition of GGT, which raised the plasma glutathione level significantly (Stipanuk et al., 2006).

Glutathione, exists mainly in the reduced state (GSH), with <5-10% of the total existing as glutathione disulfide (GSSG) under normal physiologic conditions. The GSH/GSSG redox cycle, one of the major endogenous antioxidant systems, protects cells from injurious events resulting from exposure to toxic chemicals, as well as the normal oxidative products of cellular metabolism.

Normal turnover of GSH

The normal turnover of GSH in human adults has been estimated to be approximately 40 mmol/day, which are slightly greater than Cys turnover in the body protein pool. Although, GSH turnover returns Cys to the Cys pool, and oxidized glutathione can be reduced back to GSH,

some Cys is irreversibly lost through excretion of glutathione or its metabolites (*e.g.*, mercapturic acids). Thus, some GSH accumulated extracellularly is excreted with the urine. In addition to GSH, Cys moieties and glutamyl-cysteine are also excreted in the urine (Stipanuk et al., 2006).

Extracellular degradation is the basis for considering glutathione a physiological reservoir for Cys. In mammals, glutathione is maintained at a high intracellular concentration (0.5 to 6 mM) and a very low extracellular concentration (0.25 to 50 μM), with a half-life in blood of 1 to 2 min (Yang et al., 1994; Rice and Russo-Menna, 1998; Chen et al., 2008).

The concentration in the brain is in a range of 1 to 3 mM. The glutathione concentration in brain astrocytes appears to be higher than that in neurons. Extracellular GSH has been monitored in brain by microdialysis (Yang et al., 1994; Lada and Kennedy, 1997) and these studies indicate that brain cells are able to release glutathione. In brain slices, its release was induced by depolarization and it was concluded that neurons are the glutathione-releasing cell type in brain. Glutathione released by brain cells, may contribute to the maintenance of the glutathione levels in the cerebrospinal fluid (Rice and Russo-Menna, 1998; Drigen, 2000; Ghersi-Egea et al., 2006)

Increasing Glutathione levels in brain

If orally administered, GSH is hydrolyzed by dipeptidase in the gastrointestinal tract. Intravenously administered GSH is also rapidly eliminated, with a half-life of only 7 min, by reaction with γ-GT. Only 0.5% of radiolabeled GSH administered by intra-carotid injection was detectable in brain extracts. It is generally considered to be difficult for GSH to cross the blood-brain-barrier, although there are some reports describing a so-called "GSH transporter" (Aebi et al., 1991; Jain et al., 1991; Kannan et al., 1996; Zeevalk et al., 2007). However, systemic administration of NAC can deliver Cys to the brain, thereby raising the GSH level in the CNS. There are, in fact, reports of systemic NAC administration being beneficial in animal models with neurological disorders (Aoyama et al., 2006, 2008).

Role of NAC

NAC is currently used in clinical medicine and was introduced as a mucolytic agent in the 1960s. Almost 50 years of experience in the prophylaxis and therapy of a variety of clinical conditions, mostly involving GSH depletion and alterations of the redox status, have established the safety

of this drug, even at very high doses and for long-term treatments. NAC has an impressive array of mechanisms and protective effects towards DNA damage and carcinogenesis, which are related to its nucleophilicity and antioxidant activity (which influence the toxicokinetics of xenobiotics) (De Flora et al., 2001). Apart from its medicinal use in patients with respiratory diseases, it also has found therapeutic use in the treatment of acetaminophen overdose (Ballatori et al., 1998) and in raising GSH levels in HIV patients (Bridgeman et al., 1991; Zafarullah et al., 2003).

NAC is not toxic to humans, the $t_{1/2}$ for NAC in blood plasma is approximately 2 hours and the volume of distribution is 0.33 L/kg, indicating its distribution mainly to extracellular water (Ballatori et al., 1998). It is rapidly eliminated in urine, with approximately one-third excreted during the first 12 hr after administration (Borgstrom et al., 1986). In urine is present mostly as the symmetrical disulfide although may be present as the mixed disulfide with Cys, and as a free thiol.

Regulation of Cysteine

The mammalian liver tightly regulates its free Cys pool. Intracellular Cys in rat liver is maintained between 20 and 100 nmol/g (to vary about 5-fold), even when sulfur amino acid intakes are deficient or excessive, while regulating Cys degradation to maintain the plasma Cys concentration is within a 2.5-fold range (between 80 and 200 mmol/L). By keeping Cys levels within a narrow range and by regulating the synthesis of glutathione, which serves as a reservoir of Cys, the liver addresses 2 opposing homeostatic requirements: the need to have adequate Cys to support normal metabolism and the need to keep Cys levels below the threshold of toxicity, because high levels of Cys are known to present cytotoxicity. Elevated tissue Cys levels should be avoided because they may lead to autoxidation of Cys to form cystine and ROS, oxidation of protein thiol groups, neurotoxicity mediated by NMDA-type glutamate receptors or membrane cystine/glutamate exchanger activity, or excess production of H_2S via desulfhydration reactions.

Catabolism of Cys is also tightly regulated via regulation of Cys dioxygenase (CDO) levels in the liver, with the turnover of CDO protein being dramatically decreased when intracellular Cys levels increase. This occurs in response to changes in the intracellular Cys concentration via changes in the rate of CDO ubiquitination and, hence, degradation. Because the response hepatic CDO to an increase in Cys or methionine load is rapid (*i.e.*, < 24 h to reach new steady-state in rats) and large (*i.e.*, > 30-fold increase in CDO protein in rats), the liver provides a substantial

safeguard against intake of excess Cys *via* the oral dietary route, particularly if dietary changes are made gradually (Stipanuk et al., 2006).

NAC as a potential chemopreventive agent

The pyrrole ring of the formed pyrroles, is an electron-rich heterocyclic, and is readily oxidized photochemically, electrochemically, or by several oxidizing agents including molecular oxygen. After autoxidize may bind to other lysine amino groups inducing crosslinks in neurofilaments responsible by 2,5-HD neuropathogenic toxicity (Genter et al., 1988; Amarnath, et al., 1994; LoPachin and Lehning, 1997). However, the inhibition of pyrroles autoxidation may be promoted by thiol compounds, knowing the high affinity of pyrroles for the sulfhydryl groups in proteins, peptides or aminoacids. NAC, a thiolic antioxidant, acts as a precursor of the natural antioxidant GSH, by direct reaction between its reducing thiol group and oxygen free radicals. GSH acts as hydrogen donor for free radicals since the S-H bond is relatively weak, and may also protect against oxidant damage *in vitro* and *in vivo* (Zhu et al., 1995; Banaclocha et al., 1997; Aoyama et al., 2008).

In vitro and *in vivo* models demonstrated the formation of stable secondary pyrrole adducts with sulfhydryl groups of Cys which may inhibit pyrrole-pyrrole crosslinking (Zhu et al., 1994, 1995, 1997; Torres et al., 2010, 2012a,b; Mateus et al., 2012).

The biological thiol GSH could exert protective effects against γ-diketone neuropathy by interrupting the cross-linking process, however, as mentioned earlier, when orally administered is hydrolyzed in the gastrointestinal tract and concerning the intravenously administration, GSH has an half-life of only 7 min, by reaction with γGT. Finally, it is generally considered to be difficult for GSH to cross the blood-brain-barrier (Jain et al., 1991; Zeevalk, et al., 2007) and the precise mechanism underlying GSH transport from blood to the brain remains unknown (Aoyama et al., 2008).

Systemic administration of NAC can deliver Cys to the brain, thereby raising the GSH level in the CNS. There are, in fact, reports of systemic NAC administration being beneficial in animal models with neurological disorders. Therefore, NAC is the simplest Cys pro-drug that can be systemically administered to deliver Cys to the brain, acting as a precursor for glutathione synthesis as well as a stimulator of the cytosolic enzymes involved in glutathione regeneration. Systemic administration of NAC reduces markers of oxidative damage and increases brain levels

of glutathione in mice (Banaclocha, 2001). Increasing the neuronal GSH level, through the administration of NAC, would prevent the progression of some neurodegenerative diseases by protecting against oxidative stress and ROS (Schulz et al., 2000; Aoyama et al., 2008).

NAC and neurodegenerative diseases

The mechanisms of NAC action at the cellular level, and the usefulness of this antioxidant are very important for the treatment of age-associated neurodegenerative diseases.

This thiol can act i) as a precursor for glutathione synthesis as well as a stimulator of the cytosolic enzymes involved in glutathione regeneration ii) by direct reaction between its reducing thiol group and reactive oxygen species. iii) preventing programmed cell death in cultured neuronal cells iv) increasing mitochondrial complex I and IV specific activities both *in vitro* and *in vivo* in synaptic mitochondrial preparations from aged mice. Systemic administration of NAC reduces markers of oxidative damage and increases brain levels of glutathione in mice (Banaclocha, 2001).

All pharmacological agents proposed for any therapeutic or prophylactic use in humans, including prevention of cancer and other mutation-related diseases, should possess some general requisites, regarding cost, practicality of use, safety and efficacy (Pendyala and Creaven, 1995). In this domain, it is noteworthy that NAC preparations have a low cost and are of practical use, being stable for years in dry form. Moreover, oral administration is compatible with long-term use. At variance with other novel molecules that are candidate chemopreventive agents, for which phase I studies are needed in order to evaluate pharmacokinetics and tolerability, the pharmacokinetics and bioavailability of NAC have been extensively investigated (Pendyala and Creaven, 1995; De Flora et al., 2001). The safety of NAC has been well established after almost 50 years of clinical experience.

1.7 METHODS FOR ASSESSING NEUROTOXICITY

To assess the neurotoxicity of a product is necessary to take measurements at different levels, which may include morphological, biochemical, electrophysiological and behavioral disorders (Markel et al., 1989; Kulig et al., 1996; Moser et al., 1997).

Neurobehavioral and pathological evaluations of the nervous system are additional components of basic research and toxicity testing of different neurotoxic products. They may express its neurological and behavioral effect in different ways and with varying specificity due to the high heterogeneity of the nervous system (Moser, 2011).

The selection of the most appropriate methods depends on the purpose of the study. Although, none of the methods allow complete information of the action of compounds on the nervous system, behavioral tests show a number of advantages that make them very useful for studying the effects of exposure to a neurotoxic product.
One of the advantages of behavioral methods is that the behavioral assays are not invasive, and can be used in long-term studies. Furthermore, is considered that changes in the behavior can be very sensitive and early indicators in chronic exposure to neurotoxic agents (Tilson, 1993a,b, 1998).

It is important to note that there is no one single functional observation battery protocol. Guidelines rather describe more general aspects and experimental tests that should be included. Over the years several protocols for behavioral assessments have been published (*e.g.*, Kulig, 1996; Moscardo et al., 2007; O'Donoghue, 1996; Moser, 2011), and each testing laboratory generally uses its own version.

1.7.1 NEUROBEHAVIORAL TESTS IN ANIMALS

The behavioral study consists of the direct observation of animal behavior after exposure to a chemical agent being one of the more direct means of documenting the changes of clinical signs (neurological and behavioral) and enabling investigate the potential neurotoxic effects of a compound.
Observations take place in the home cage and an open field arena, during which time the subject's movements, physical appearance, and reactions to various stimuli are evaluated.
Often also included are manipulations including grip and landing foot splay (Moser, 2011). An advantage to this type of screening is that a single animal may be repeatedly assessed to

determine the onset, progression, duration, and reversibility of a neurotoxic injury, in a non-invasive way.

Behavioral tests comprise of a set of observations called functional observation battery (FOB) (Sette, 1989; Moser, 2011). FOB includes numerous physiological tests, neuromuscular (*i.e.*, weakness, incoordination, gait, and tremor), sensory (*i.e.*, audition, vision, and somatosensory) and motor tests, autonomic (*i.e.*, pupil response, salivation) and thermoregulatoy functions, and activity and excitability of the CNS (Tilson, 1993a,b; Tilson et al., 1996; Cory-Slechta et al., 2001). Neurobehavioral end points are frequently used in conjunction with other measures of neurotoxicity (Hattis et al., 1996) such as neuropathology and neurochemistry in an integrated approach to assess chemicals for possible neurotoxicity (Hattis et al., 1996).

At the first-tier, simple, rapid, inexpensive methods are typically used to detect the presence of neurotoxicity. Methods are also called of screening or first-tier testing and typically consist of simple or quick tests of behavior that may be used to identify whether a chemical acts on the nervous system, and at what dose levels. Most sensory testing available for use in this screening include simple measures of locomotor activity, sensorimotor reflexes, and neurological signs. They either involves testing simple reflexes (*e.g.*, grasping, pinna reflex) or evaluation of the motor response to a variety of sensory stimuli (*e.g.*, auditory, nociceptive, somatosensory). Activity and/or reactivity measures may be automated or observed as part of the open field evaluations (*e.g.*, rearing, arousal). Other behavioral evaluations including clinical signs of tremor, convulsions, or other motor abnormalities (Tilson, 1993a,b; Moser 2011).

Second-tier testing involves more complex tests that provide a more complete description of the effects and dose-response relationships (Tilson, 1993a,b, 1998; Tilson et al., 1996), since they are more time- and resource-intensive. Learned or conditioned behaviors require training of the test subject and are focused on specific aspects of behavior (*e.g.*, short- *vs.* long-term memory). The behavior is under procedural control, and since experimental variables may be altered to increase the specificity of the test, the data may be less variable and easier to interpret. Examples of second-tier tests include discriminated conditioned response methodologies to assess specific sensory or motor dysfunction and procedures to measure chemical-induced alterations in cognitive function (Tilson, 1998). Studies concerning biological mechanism(s) of action or those

intended to determine a no observable adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) typically employ more sensitive tests. Specificity and sensitivity of effect are more desirable test attributes if the chemical is known to produce weakness, for example, and a second tier test is used to characterize the effect and to determine a no-observed adverse effect level (NOAEL), *i.e.*, distinguish between diminished ability to exert high forces versus ataxia (Cory-Slechta et al., 2001; Moser, 2011).

With the evolution of tiered testing strategies in which each stage of evaluation incorporates decision points as to whether available information is sufficient for determining the neurotoxicity of a chemical (Tilson et al., 1996). Evans and Weiss (Tilson, 2000) outlined a three-tier testing scheme, including hazard identification, characterization, and assessment of human susceptibility. They suggested assessing human susceptibility to chemicals, using methods analogous to those employed in animal studies. In 1992, the National Research Council (NRC) published a book on environmental neurotoxicology describing a three tier-testing scheme similar to that of Evans and Laties. Its included mechanistic rather than human studies in the third tier. A three-tier testing strategy was recently endorsed by the European Chemical Industry Ecology and Toxicology Centre (ECETOC, 1992; Tilson, 2000).

The behaviors measured in FOB procedures may be sorted by domains of neurological functions, although there is often overlap since many tests tap more than one domain and these domains do not necessarily map to specific regions of the nervous system (Moser, 2011). Some autonomic endpoints may be assessed by observation (*e.g.*, salivation, lacrimation, pupil size), while others (*e.g.*, respiration, heart rate) may require specific instrumentation. Neuromuscular ability and coordination may be measured using any number of tests ranging from evaluations of gait and posture, to palpations of muscle tone or extensor strength, to instrumental tests of hindlimb and forelimb grip strength, to monitoring of righting or proprioceptive responses. Most sensory testing available for use in first-tier screening involves either testing simple reflexes (*e.g.*, grasping, pinna reflex) or evaluation of the motor response to a variety of sensory stimuli (*e.g.*, auditory, nociceptive, somatosensory). Activity and/or reactivity measures may be automated, or observed as part of the open field evaluations (*e.g.*, rearing, arousal). Other behavioral evaluations including clinical signs of tremor, convulsions, or other motor abnormalities are an

important aspect of neurological testing. Comparisons of chemical effects on multiple FOB endpoints and across functional domains aid in data interpretation.

1.7.1.1 Neurobehavioral screening in rodents

Neurobehavioral evaluations are an important of testing for the neurotoxic potential of chemicals. Observations during toxicity studies or specialized neurotoxicity studies can provide information important for identifying and/or characterizing neurotoxic effects. A neurobehavioral test battery can be composed of a variety of endpoints, usually chosen to asses an array of neurological functions, including autonomic, neuromuscular, sensory, and excitability.
The protocols can be then divided into observational assessments and manipulative tests. Each is further subdivided into specific tests or endpoints (Table 1.7).

These various endpoints may be combined into a battery of tests for neurobehavioral screening and should be used in the context of a broad neurobehavioral test battery.
For all these screening batteries, clearly defined protocols are critical to good experimentation (Moser, 2011). The use of rating scales also allows semi-quantitative assessment of the data that would otherwise have subjective evaluation (Weiss, 1988). This approach to the behavior is often more sensitive than a simply listing behaviors as ‘‘normal’’ or ‘‘abnormal’’ in which case it is critical to have working definitions of what is ‘‘normal’’. Likewise, detailed descriptions of behaviors are more informative than colloquial or nonspecific terms. Researchers conducting these tests must be ‘‘blind’’ or unaware of the animal’s treatment, so as not to introduce bias, however unintentional, into the data.

Table 1.7 Endpoints in a Neurobehavioral Screening Battery.

Observational Assessments	Manipulative Tests
Activity levels:	**Neurologic reflexes/reactions:**
Home-cage observations	Pupil response
Open-field observations	Palpebral reflex
Rearing	Pinna reflex
Reactivity/excitability:	Extensor thrust reflex
Reactivity	**Neuromuscular test and postural reactions:**
Arousal	Grip strength
Gait and postural characteristics:	Landing food splay
Gait descriptions	Hopping
Postural descriptions	Righting reaction
Involuntary/abnormal motor movements:	**Sensory responses:**
Tremors	Visual test: approach response
Fasciculations	Visual test: visual placing
Clonus	Somatosensory test: touch response
Tonus	Auditory test: click response
Stereotypy	Nociceptive test: tail/toe pinch
Bizarre behaviors	Nociceptive test: flexor reflex
Clinical signs:	Proprioceptive positioning test
Lacrimation	Olfactory test
Salivation	
Hair coat	
Palpebral closure	
Ocular abnormalities	
Muscle tone/mass	

(Adapted from: Moser V.C. Current Protocols in Toxicology, John Wiley & Sons, Inc, 1999).

These various endpoints may be combined into a battery of tests for neurobehavioral screening and should be used in the context of a broad neurobehavioral test battery.

Training is also extremely important, including careful study of the protocols and considerable practice. Observers must be taught the basics of experimental design and good laboratory techniques, understand normal behavioral repertoires and factors that can alter behaviors, be at ease handling laboratory animals, and be certified with tests using positive controls (Slikker et al., 2005; Moser, 2011).

1.7.2 STUDIES IN HUMANS

Solvents are ubiquitous in industrial societies in a wide range of processes. Clinicians are therefore likely, in daily practice, to see patients who are exposed to these agents either occupationally or through contaminated environments since the assessment of neurotoxicity begins with a clinical evaluation of the patient, including medical history and a standard neurological examination (White and Proctor, 1997; Bull, 2007).

The associated symptoms after contact with these agents are generally related to the functioning of the central (CNS) or peripheral nervous system (PNS). In many cases, such symptoms are fleeting and resolve after cessation of exposure. However, long-term chronic exposure or high-dose acute exposures can produce longer-lasting effects characterized by cognitive and behavioral changes. In some cases, the symptoms may fully or largely resolve with time, whereas in others, permanent CNS or PNS damage may occur (Chang et al., 1993).
Many studies have demonstrated the neurotoxicity of organic solvents through the use of neuroimaging, neurophysiological and neuropsychological assessment techniques (Baker and Seppäläinen, 1986; Baker, 1994). These techniques reveal neurotoxicant effects in workers who are not clinically ill. The effects have been referred to as latent, subclinical, or preclinical (Hänninen, 1971; Baker et al., 1985; Feldman and White, 1996).

1.7.2.1 Clinical assessment

When the doctor suspects that possible nervous-system disorder as a result of solvent exposure, several special clinical examinations may be useful in defining the patient’s condition (Table 1.8). As in any investigation, these examinations are most helpful if done by clinical specialists who have experience in examining patients with disorders related to occupational or environmental exposure to neurotoxicants.

The CNS depression and psychomotor or attentional deficits are generally resulted of exposure to organic solvents. The patient may present with complaints of fatigue, irritability, confusion, or depression, and may describe memory difficulties (Baker et al., 1985; Feldman and White, 1996; White and Proctor, 1997).

When PNS function is affected, initially the patient may exhibit the following symptoms: intermittent tingling and numbness, with progression to an inability to perceive sensation and muscle weakness (Yokoyama et al., 1990; Smith and Albers, 1997).

Neurological examinations can be used to define solvent-related neurological abnormalities of CNS or PNS function and to exclude other neurological explanations for the patient's symptoms. Laboratory tests of nervous-system function can be useful in confirming clinical evidence of CNS or PNS disease. For example, nerve-conduction studies and electromyelography (Seppalainen et al., 1978) can help to confirm a suspected solvent-induced peripheral neuropathy. Computed tomography (CT) and magnetic resonance imaging (MRI) can be used to detect the atrophic changes in the frontal lobes and cerebellum as well as white-matter lesions that have been described after exposure to certain solvents (Yamanouchi et al., 1995; Ridgway et al., 2003). Neuropsychological testing can also be helpful in defining any intellectual and affective deficits that may be associated with exposure (Hänninen et al., 1976; Chang, 1987).

Questionnaires and biological monitoring are generally the most feasible approaches for the clinician. In biological monitoring for specific solvents, the solvents themselves or metabolites can sometimes be measured in the patient's blood, urine, hair, fat, or breath. This approach is the most accurate in assessment of internalized dose, but its use can be limited by the expense of sampling and measurement and concern about the accuracy of the measure (Thorne, 2008).

Chronic exposure can be associated with permanent cognitive changes (Baker and Seppäläinen, 1987; Hänninen et al., 1976). Typically, these changes in adults are limited to specific behavioral domains, including attentional capacity, executive function (the ability to deploy strategies for problem-solving and to organize responses to novel stimuli), visuospatial skills (analysis and integration of visual arrays), short-term memory, and mood/affect (White and Proctor, 1997).

In the following Chapter it is presented the main and specific objectives to be achieved in this thesis.

CHAPTER 2

OBJECTIVES

Control and prevention of human exposure to neurotoxic chemicals is an important area of research that certainly contributes to the promotion of human health. Thus, it is important to control the exposure to neurotoxicants and to study protector agents and/or antagonist agents, which may reduce the pathologies due to environmental/occupational exposure.

In this context, this thesis aims to implement measures to control and prevent the neurotoxicity induced by 2,5-hexanedione studying alternative biomarkers of exposure and effect.

Main Objective

To select biomarkers to control cumulative exposure to n-hexane and predict the neurotoxic effects induced by its neurotoxic metabolite 2,5-HD.

Specific objectives

- To identify the pyrroles compounds excreted in urine in rats exposed to 2,5-HD.
- To determine which of the identified and quantified pyrroles can be used as indicators of cumulative exposure to 2,5-HD exposure.
- To investigate if the formed pyrrole adducts may be proposed as predictive biomarkers of 2,5-HD neurotoxicity.
- To study the mechanism of interference of NAC in 2,5-HD neurotoxicity.
- To investigate the presence of the identified pyrroles in human urine of an healthy and patients with neurodegenerative diseases.

To achieve these objectives we performed the following experiments:

In Chapter 3

Synthesis and characterization of standard pyrrole compounds, identification and quantification of aminopyrrole adducts and aminopyrroles thiol conjugates in rat urine by ESI-LC-MS/MS.

In Chapter 4

An *in vivo* model was used to investigate if the identified pyrroles could be selected as predictive biomarkers of effect by correlating the changes in urinary 2,5-HD and selected identified pyrroles with the changes in motor activity assays in rats treated with 4 doses of 2,5-HD and 2,5-HD+NAC.

In Chapter 5

A new *in vivo* assay was performed treating the rats with 12 doses of 2,5-HD and 2,5-HD+NAC to evaluate the protector/toxicity binomium induced by NAC on 2,5-HD neurotoxicity.

Along the 12 doses treatment was determined the changes on selected urinary biomarkers and the motor activity performance.

At the end of the experiment was studied the mechanism of interference of NAC on 2,5-HD neurotoxicity by measuring Cysteine and GSH in rat brain.

In Chapter 6

We performed a preliminary study to investigate the presence of the identified pyrroles in human urine of an healthy and a sick populations.

CHAPTER 3

ALTERNATIVE BIOMARKERS OF n-HEXANE EXPOSURE

3.1 INTRODUCTION

Biologic monitoring of occupational exposure to n-hexane has been routinely evaluated by determining the urinary levels of total 2,5-HD (Perbellini et al., 1990). More recently the determination of free 2,5-HD has been used as an alternative or complementary biomarker of n-hexane exposure (Manini et al., 2004, 2006). However, as urinary 2,5-HD can only be a measure of recent exposure and the most common situation is the chronic exposure to this solvent (Toxicological review n-hexane, EPA, 2005), the study of other biomarkers is of great interest.
In vitro and *in vivo* models demonstrated that 2,5-HD forms pyrrole adducts with amino groups in proteins, and pyrrole derivatives (PLS) were quantified in urine, hair and serum using the Ehrlich's reagent, until now the most used method to quantify pyrroles compounds (Kessler et al., 1990; Johnson et al., 1995; Santos and Batoréu, 1991, 1993; Yin et al., 2013a,b). However, this colorimetric method quantifies all the compounds that have the pyrrole ring (Mattocks and White, 1970; DeCaprio et al., 1982) thus, it is not a specific method to analyze only the pyrroles resulting from 2,5-HD exposure. On the other hand, previous studies (Graham et al., 1995; Costa, 1996; Mateus et al., 2001) indicate that PLS could be a good indicator of cumulative exposure to 2,5-HD. Therefore, it was imperative to develop a method to determine the specific pyrroles formed after n-hexane exposure.

In this chapter, our goal was to identify and quantify the pyrrole compounds excreted in urine to be used as biomarkers of repeated exposure to n-hexane. To achieve this objective we performed the following experiments:

i) Synthesis and identification of one aminopyrrole compound and its thiol conjugates, to be used as standards;
ii) Identification of pyrrole compounds excreted in urine of rats treated with 3 doses of 2,5-HD;
iii) Quantification of identified compounds.

The synthesis of target pyrrole compounds and their identification by ESI-LC-MS/MS was based on previous work (Zhu et al., 1994, 1995, 1997; Torres et al., 2010, 2012a,b, 2014; Mateus et al., 2012).

The compounds synthesized were DMPN, DMPN NAC and DMPN GSH (Fig. 3.1). The synthesis of these standards was essential for the development of our work, since by now they are not commercialized.

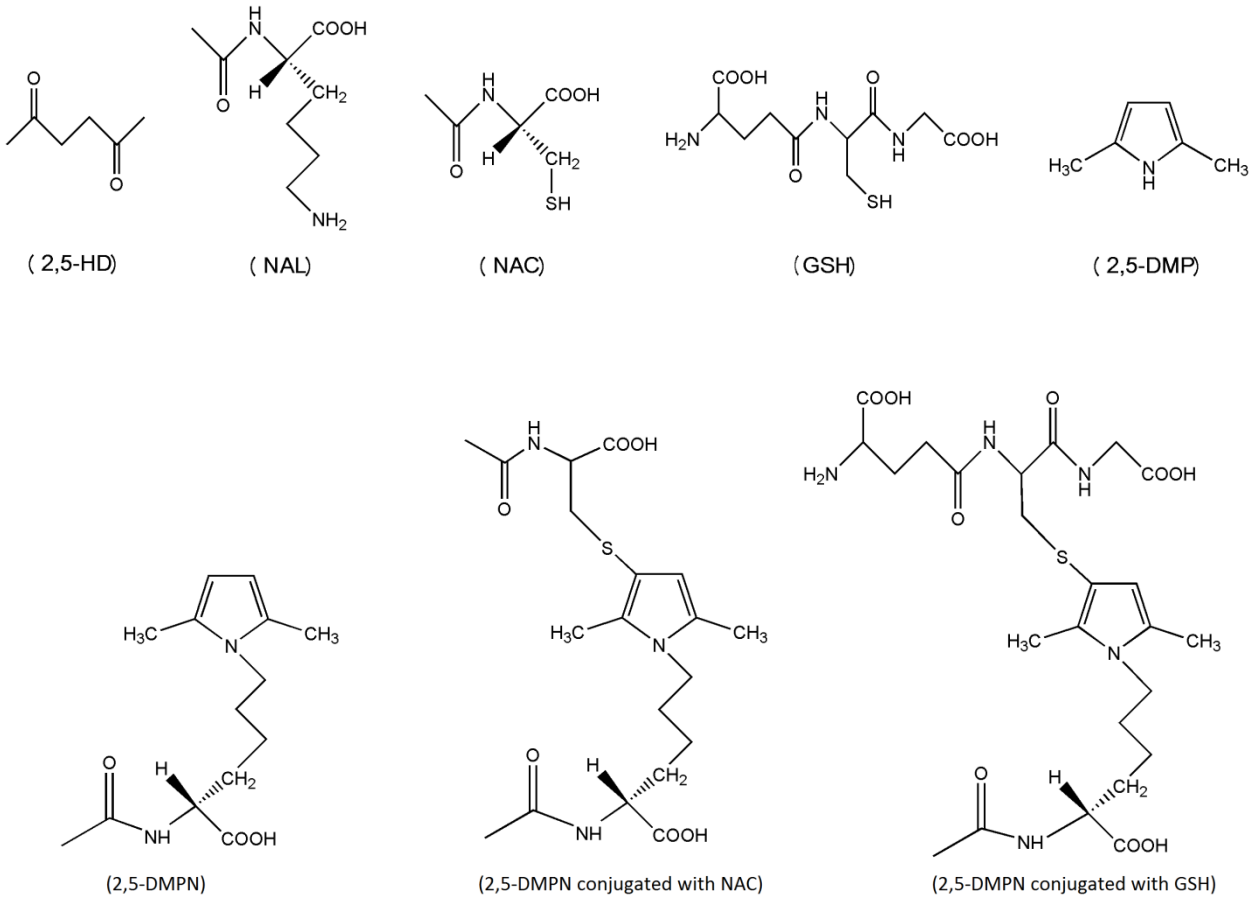


Figure 3.1 Chemical structure of the pyrrole derivatives synthesized: Nα-Acetyl-6-(2,5-dimethylpyrrol-1-yl)norleucine (**DMPN**), Nα-Acetyl-6-[3-(N-acetylcystein-S-yl)-2,5-dimethylpyrrol-1-yl]norleucine (**N-acetylcysteine conjugate of DMPN** or **DMPN NAC**) and 2-Acetylamino-6-{3-[2-(4-amino-4-carboxy-butyrylamino)-2- (carboxymethyl-carbamoyl)-ethylsulfanyl]-2,5-dimethyl-pyrrol-1-yl}-hexanoic acid (**GSH conjugate of DMPN** or **DMPN GSH**). 2,5-HD, NAL, NAC and GSH are reagents. 2,5-DMP is a pyrrole compound standard.

The experimental work includes the synthesis and chemical characterization of the products formed upon incubation of 2,5-HD with Nα-acetyl-L-lysine (NAL) in the presence or absence of Nα-acetyl-L-cysteine (NAC) or glutathione (GSH).

The synthesis of pyrrole compounds was firstly followed through the formation of a chromophore (Fig. 3.2) between the pyrrole ring and 4-dimethylaminobenzaldheyde (Ehrlich's reagent). Despite the lack of method specificity, it is still widely used due to its high sensitivity, low cost, easy and quick to perform.

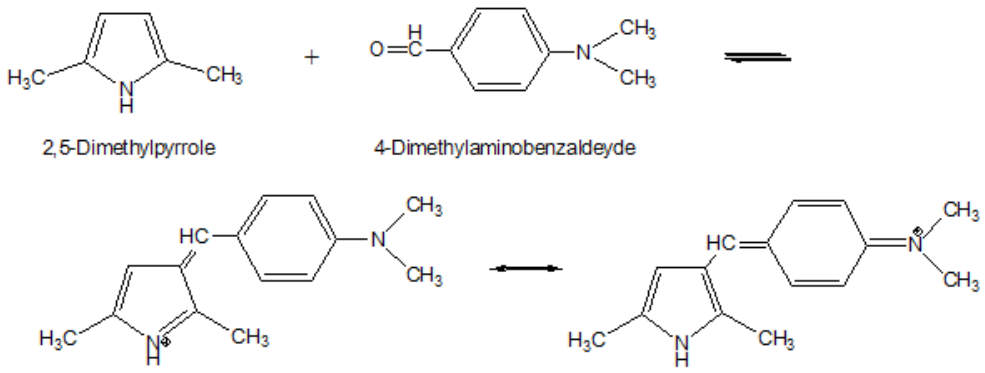


Figure 3.2 Scheme reaction of 2,5-DMP with *p*-DMAB. (Adapted from Ogata et al., 1991).

After an Ehrlich positive reaction, HPLC combined with diode array and electrospray ionization mass spectrometric detection was used to identify the different synthesized pyrrole compounds.

3.2 EXPERIMENTAL PROTOCOL

3.2.1 SYNTHESIS OF STANDARD COMPOUNDS

For each synthesis, preliminary studies were performed with different concentrations of reagents, different reaction times and under different atmosphere conditions, to select conditions which had the highest performance.

The following experiments describe the conditions selected for each synthesis. All synthesis were monitored during 48 h.

3.2.1.1 DMPN

Nα-acetyl-6-(2,5-dimethylpyrrol-1-yl)norleucine, (DMPN), was synthesized by incubating Nα-acetyl-L-lysine (100 mM) with 2,5-HD (100 mM), **reaction I**, in a two-necked flask immersed in a thermostatic bath.
The reaction mixture was kept at 37 °C, with stirring, in the absence of air (under nitrogen) and of light. Hereafter these conditions will be referred as standard incubation conditions.

3.2.1.2 DMPN NAC CONJUGATE

Nα-acetyl-6-[3-(N-acetylcystein-S-yl)-2,5-dimethylpyrrol-1-yl]norleucine (N-acetylcysteine conjugate of DMPN) was synthesized by incubating Nα-acetyl-L-lysine (100 mM) and Nα-acetyl-L-cysteine (10 mM) with 2,5-HD (100 mM), **reaction II**. The resulting mixture was kept under standard incubation conditions.

3.2.1.3 DMPN GSH CONJUGATE

The pyrrole compound, 2-Acetylamino-6-{3-[2-(4-amino-4-carboxy-butyrylamino)-2-(carboxymethyl-carbamoyl)-ethylsulfanyl]-2,5-dimethyl-pyrrol-1-yl}-hexanoic, (GSH conjugate of DMPN), was synthesized by incubating Nα-acetyl-L-lysine (100 mM) and GSH in reduced form (10 mM) with 2,5-HD (100 mM), **reaction III**. The resulting mixture was stirred under the same standard incubation conditions referred before.

3.2.2 INSTRUMENTAL METHODS

3.2.2.1 Spectrophotometric Method

A Hitachi U-2000, UV–Vis spectrophotometer was used to detect the synthesized pyrrole after reaction of one aliquot (0.1 mL) of the product of reaction under study, with 1.9 mL of Ehrlich's reagent (3% 4-dimethylaminobenzaldheyde in 40 mL of methanolic 14% boron trifluoride and

60 mL of ethanol) (Mattocks and White, 1970). Absorption values were measured at 526 nm (*i.e.* at the wavelength corresponding to spectrum maximum absorption) (Mateus et al., 2001). Each pyrrole was quantified using 2,5-DMP calibration curves.

3.2.2.2 Liquid Chromatography and Mass Spectrometry Method

A Waters Alliance 2695 (Waters®, Ireland) LC system equipped with a quaternary pump, solvent degasser, auto sampler, column oven and a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) with an electrospray ionization source (ESI), was used to obtain structural information for the identification of pyrrole derivatives.

The ion source temperature was kept at 120ºC and capillary voltage was 3.5 kV. Argon was used as collision gas. In addition, a Photodiode Array Detector Waters 996 PDA (Waters®, Ireland) was used to scan wavelength from 210 to 600 nm. Data acquisition, processing and instrument control was performed by Micromass MassLynx version 4.1 Software.

The LC separation was performed on a reversed-phase column (LiChrospher® RP18, 125 × 4; 5.0 µm; Merck®) at 35ºC using an injection volume of 5 µL.

The mobile phase consisted of ammonium acetate buffer 0.02 M (pH 5.2) (A) and acetonitrile (B) at a flow rate of 0.30 mL/min. A gradient of eluents was used for analysis, starting at 100% of A, and down to 30% A for 15 min. The conditions were kept during 1 min at 30% A and after the column were equilibrated with 100% eluent A for 5 min.

The identification and characterization of pyrrole compounds was performed on a system LC Waters Alliance 2695 (Waters®, Ireland) equipped with the following elements: Quaternary pump, Solvent degasser, Auto sampler, Column oven and a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland). Photodiode Array Detector Waters 996 PDA (Waters®, Ireland). Micromass MassLynx version 4.1 software.

Individual solutions of each standard compound (2,5-HD, NAL, NAC and 2,5-DMP), and the solutions with the synthesized compounds (DMPN, DMPN conjugates: DMPN NAC and DMPN GSH) were infused into the mass spectrometer and scanned in the positive ion mode in order to achieve the best conditions of analysis (cone and capillary voltage) corresponding to higher signal/noise ratio to detect the molecular ion.

A spectrum for each compound was obtained in the *m/z* range from 40 to 600. After, Daughter Scan mode was used in order to choose the best collision energy, adequate to obtain a fragmentation spectra characteristic of each compound, to maximize the two main product ions signals. For the quantification, the mass spectrometer was operated in multiple reactions monitoring (*MRM*) mode. In this work one transition (*MRM*1) was used for quantification and another one (*MRM*2) for confirmation. The use of two transitions gives 4 identification points, corresponding to one precursor (1 point) and two product ions (3 points). The specificity of the method of analysis was guaranteed based on the comparison of: (1) the retention time variation of the standards and the target analytes in the samples, and (2) *MRM*1/*MRM*2 ratios. For peaks detected in samples, this ratio should not be outside the ±20% range, considering results obtained with the standards analyzed in the same conditions. These criteria are according to the Commission Decision 2002/657/EC.

Matrix effect during analyte ionization may cause suppression or enhancement of the analyte signal. This effect was evaluated for 2,5-DMP commercial standard, by comparing the signal obtained from the calibration curves prepared using water as solvent or urine from control rats. The ion suppression (expressed in percentages) was quantified as 1 minus the ratio between the slope of the curve obtained with H_2O and the slope of the curve with urine from control rats. If the result is positive, we have an ion suppression of the analyte signal. If it is negative, we have an ion enhancement.

Previously published work describing *in vitro* assays allowed a comparative study with the chromatograms of the synthesized compounds (Zhu et al., 1994, 1995, 1997).

3.2.3 ANIMALS

Male Wistar rats (220 ± 25 g) from Charles River Laboratories® (Barcelona), were housed at controlled temperature, humidity and a 12-h light/dark cycle. Throughout the one week acclimatization period, and during the exposure, the general conditions of the animals were checked daily. Animals had free access to water and rat standard food (Letica Ref. IPM-R20).

All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

3.2.4 EXPOSURE OF RATS AND URINE COLLECTION

After the acclimatization period, the animals were randomly assigned to two groups (n=7): Group I – 400 mg 2,5-HD/kg bw/48-h (ip injection); Group II – saline solution (ip injection in the same days of Group I).

2,5-HD was made ip administrated due to the ease of administration compared with other parenteral routes. The γ-diketone was dissolved in a sterile saline solution and was administered to the animals with a 48-hour interval.

The concentration of the 2,5-HD solution was calculated in order that the volume of the 2,5-HD by ip administration, was approximately 1 mL. In Table 3.1 is shown the experimental design of animal assay.

Table 3.1 Animal assay protocol.

Group	Treatment solution[a]	Doses
HD (**I**)	400 mg 2,5-HD/kg bw/48-h	3 (HD)
Control (**II**)	Sterile saline/48-h	3 (saline)

(a) All treatment solutions were prepared freshly on a daily basis. 2,5-HD and saline were administered *via* ip injection (7 animals/group).

During the 24 hours urine collection, animals were kept in metabolic cages provided with urine-feces separator. All urine samples were collected over ice and out of light. All urines were centrifuged to remove particulate matter and stored at –80ºC until analysis.

Before analysis by ESI-LC-MS/MS the urine samples were filtered on a 0.20 µm membrane (CHROMAFIL® Xtra) (Moriwaki et al., 2000; Manini et al., 2004).

3.3 RESULTS

3.3.1 DETECTION OF PYRROLE COMPOUNDS BY SPECTROPHOTOMETRIC METHOD

As previously stated, all reactions were followed by Ehrlich's reaction, to confirm the formation of pyrrole compounds.

Figure 3.3 shows pyrrole concentrations expressed in equivalent of 2,5-DMP synthesized in reaction I and II in function of reaction time. The presence of NAC increases markedly the detection of levels of equivalents of 2,5-DMP.

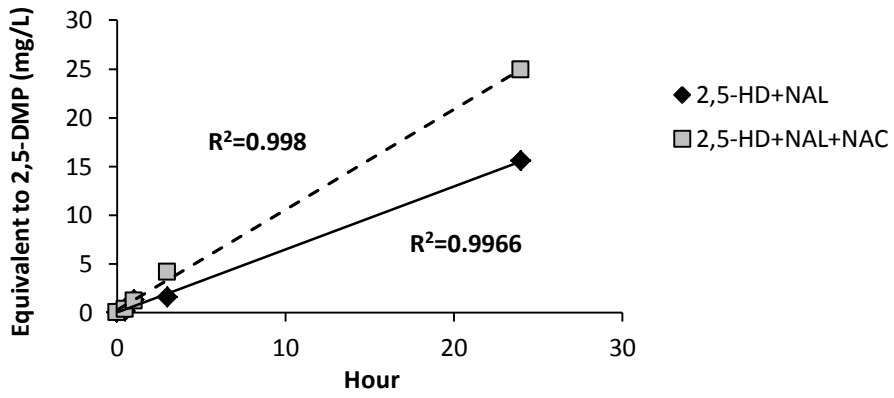


Figure 3.3 Total pyrrole concentrations, reactions I and II. Reaction I: 2,5-HD+NAL and reaction II: 2,5-HD+NAL+NAC, analyzed by spectrophotometric method. The concentration of pyrrole compounds is expressed as equivalent of 2,5-DMP (mg/L). Values are expressed as mean values (n=3).

3.3.2 IDENTIFICATION OF STANDARDS: DMPN, DMPN NAC, DMPN GSH AND 2,5-DMP, BY ESI-LC-MS/MS

3.3.2.1 Optimization of MS Conditions

The optimum conditions of analysis for each standard were selected, in order to have a higher signal of the precursor ion (see Table 3.2). Cone Voltage was 20 V to DMPN NAC, and 30 V to other compounds. Collision energy was 15 eV to 2,5-DMP and 20 eV to other compounds.

This study was performed by direct infusion of individual solutions of standards in the ion source. For all the compounds, the mass of the precursor ion corresponds to the mass of the corresponding protonated molecule $[M+H]^+$.

The molecular structure of the synthesized compounds was confirmed from the analysis of the mass spectra and fragmentation pattern obtained for each compound. The proposed chemical structures of each product ion are presented in figures 3.4, 3.5 and 3.6 and their structures are according to other authors Zhu et al., (1994, 1995). The following figures also show the mass spectra obtained after fragmentation of the precursor ions, using the Daughter Scan acquisition mode, as well as the MS/MS spectrum of the commercial standard for the 2,5-DMP (Fig. 3.7).

MS/MS spectrum of DMPN

In Fig. 3.4 is shown the fragmentation of the precursor ion $[M+H]^+$ at *m/z* 267 corresponding to pyrrole compound DMPN.

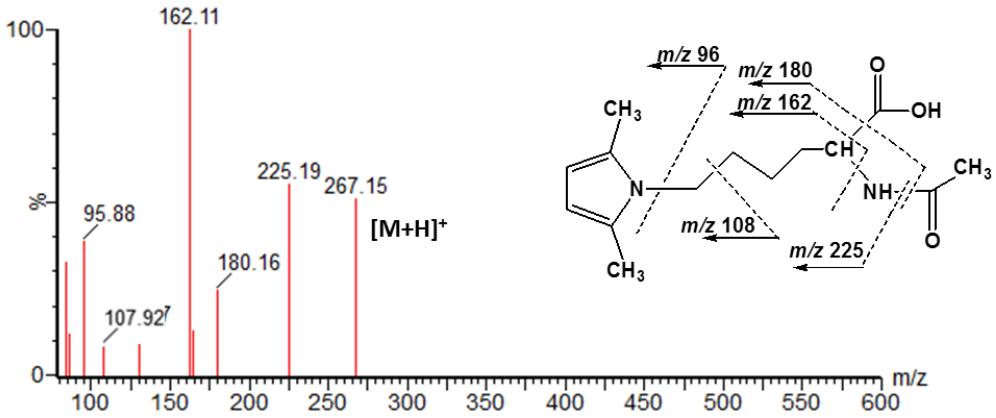


Figure 3.4 MS/MS spectrum of synthesized pyrrole compound, DMPN, obtained after the fragmentation of the molecular ion *m/z* 267.

Fragmentation of DMPN at *m/z* 267 originated several fragments: an ion fragment at *m/z* 225 corresponding to the loss of *N*-acetyl group $[M-((C=O)CH_3)]^+$, another at *m/z* 180 corresponding to the loss of both groups, carboxylic acid and *N*-acetyl, $[M-(CO_2H)-((C=O)CH_3)]^+$, and also *m/z* 162 corresponding to the loss of carboxylic acid and ethanamide derived group, $[M-(CO_2H)-(NH(C=O)CH_3)]^+$. This product ion at *m/z* 162 is the

one with higher intensity. The ion at *m/z* 108 results from the cleavage of the bond between the two carbons of the lysine near *N*-pyrrole.

For quantification of this compound the transition 267>162 (*MRM*1) was used as the transition of quantification, and 267>225 (*MRM*2) as transition of confirmation as presented in Table 3.2.

MS/MS spectrum of DMPN NAC

The mass spectrum of DMPN NAC is shown in Fig. 3.5. The fragmentation of the precursor ion $[M+H]^+$ at *m/z* 428 originated several fragments, among them mention should be made to *m/z* 299 and *m/z* 410.

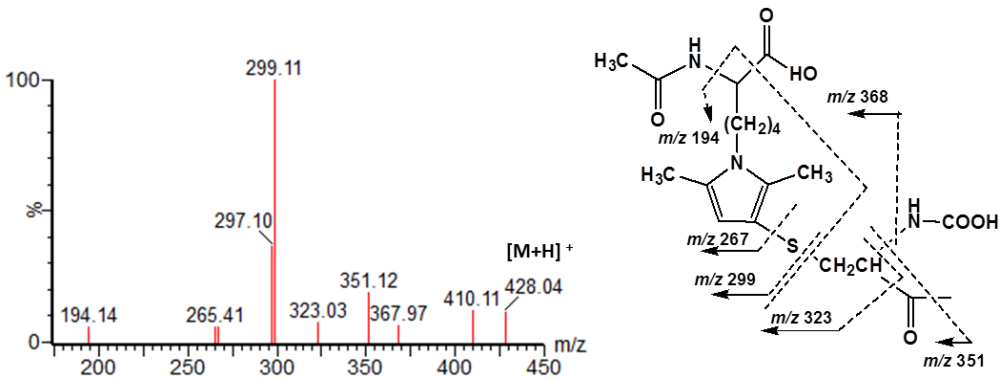


Figure 3.5 MS/MS spectrum of synthesized pyrrole compound, DMPN NAC, obtained after the fragmentation of the molecular ion *m/z* 428.

The fragment at *m/z* 299 corresponds to the cleavage of the bond between the sulfur and carbon of Cys. This fragment is the one with greater intensity. The ion at *m/z* 410 results from loss of water molecule from the molecular ion $[M{-}H_2O]^+$. For *m/z* 368 we propose that protonated molecule loss the derivated carbamic acid $[M{-}(NHCO_2H)]^+$. Ions at *m/z* 194 and *m/z* 267 were also originated from the cleavage of the two C–S bonds.

For quantification of DMPN NAC the transition 428>299 (*MRM*1) was used as the transition of quantification, and 428>410 (*MRM*2) as transition of confirmation, as presented in Table 3.2.

MS/MS spectrum of DMPN GSH

Figure 3.6 presents the mass spectra of the synthesized compound, DMPN GSH. From the fragmentation of the precursor ion $[M+H]^+$ at *m/z* 572, fragments *m/z* 443 and *m/z* 297 were the main obtained. The first results from the cleavage at N atom and 2-amino-5-oxo-pentanoic acid group, $[M-((C=O)(CH_2)_2CH(NH_2)CO_2H)]^+$. Fragment at *m/z* 297 was obtained from the cleavage at C–S bond furthest from the pyrrole ring, led to the formation of thiol-pyrrole adduct. This fragment ion is similar to those of DMPN NAC, suggesting that thiol group of GSH and NAC was directly bound to the pyrrole ring.

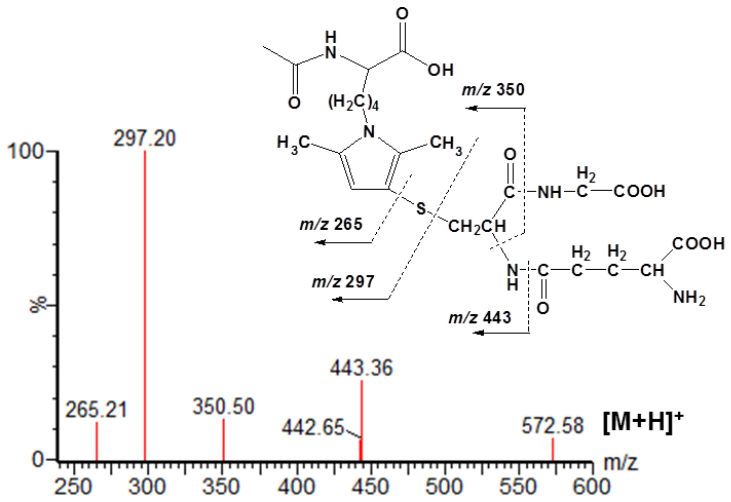


Figure 3.6 MS/MS spectrum of synthesized pyrrole compound, DMPN GSH, obtained after the fragmentation of the molecular ion *m/z* 572.

MS/MS spectrum of Commercial Standard Compound 2,5-DMP

In Fig. 3.7 is shown the fragmentation of the precursor ion $[M+H]^+$ at *m/z* 96, corresponding to pyrrole compound 2,5-DMP (2,5-dimethylpyrrole).

Fragmentation of 2,5-DMP at *m/z* 96 originated several fragments: an ion fragment at *m/z* 81 corresponding to the loss of methyl group $[M-CH_3)]^+$, another at *m/z* 80 corresponding to the loss of a hydrogen atom, $[M-(H)CH_3)]^+$. When the precursor ion loses two methyl groups yields

the fragment mass *m/z* 67. The product ion at *m/z* 81 is the one with higher intensity. For quantification of this compound transitions 96>80 (*MRM*1) and 98>81 (*MRM*2) were chosen as the transition of quantification and confirmation as presented in Table 3.2

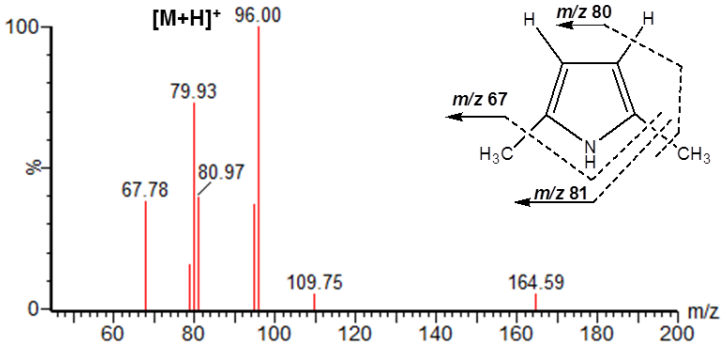


Figure 3.7 MS/MS spectrum of standard pyrrole compound, 2,5-DMP, obtained after the fragmentation of the molecular ion *m/z* 96.

In Table 3.2 are presented the principal ion intensity values obtained in the MS/MS spectra of pyrrole compounds synthesized and 2,5-DMP standard.

Table 3.2 Optimized conditions for studied compounds.

Rt (min)	**Compound**	**Precursor ion (*m/z*), $[M+H]^+$**	**Products ions, *m/z* (relative intensity)**	***Cone Voltage (V)***	***Collision Energy (eV)***	***MRM*1 transition**	***MRM*2 transition**	***MRM*1/*MRM*2 (8<n<10; RSD)**
10.7	**DMPN GSH**	572	**443**$_{(28)}$, **97**$_{(100)}$, 350$_{(13)}$, 265$_{(11)}$	30	20	572>297	572>443	2.29 (4.9%)
11.1	**DMPN NAC**	428	**410**$_{(12)}$, 351$_{(19)}$, **299**$_{(100)}$, 97$_{(36)}$, 194$_{(6)}$	20	20	428>299	428>410	2.23 (6.0%)
13.6	**DMPN**	267	**225**$_{(19)}$, 180$_{(37)}$, **162**$_{(100)}$, 108$_{(37)}$, 96$_{(65)}$	30	20	267>162	267>225	2.34 (3.4%)
18.7	**2,5-DMP**	96	96$_{(100)}$, 95$_{(37)}$, **80**$_{(73)}$, **81**$_{(40)}$, 79$_{(15)}$, 68$_{(36)}$	30	15	96>80	96>81	3.42 (9.0%)

3.3.2.2 Detection of the synthesized compounds using MRM

DMPN

Figure 3.8 shows the detection of the compound at the studied conditions. This compound was detected at 13.7 min.

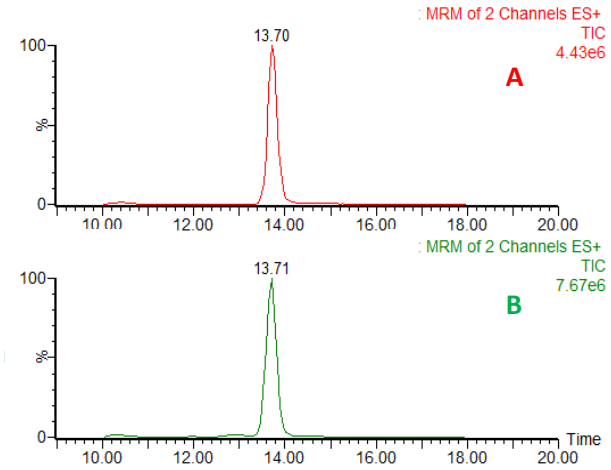


Figure 3.8 Chromatograms obtained, in MRM mode of compound DMPN: (A) 24 hours and (B) 48 hours of incubation reaction.

The formation of the compound DMPN was followed at the beginning and monitored at 2 different reaction times, 24 and 48 hours of incubation (Fig. 3.9) using Diode Array.

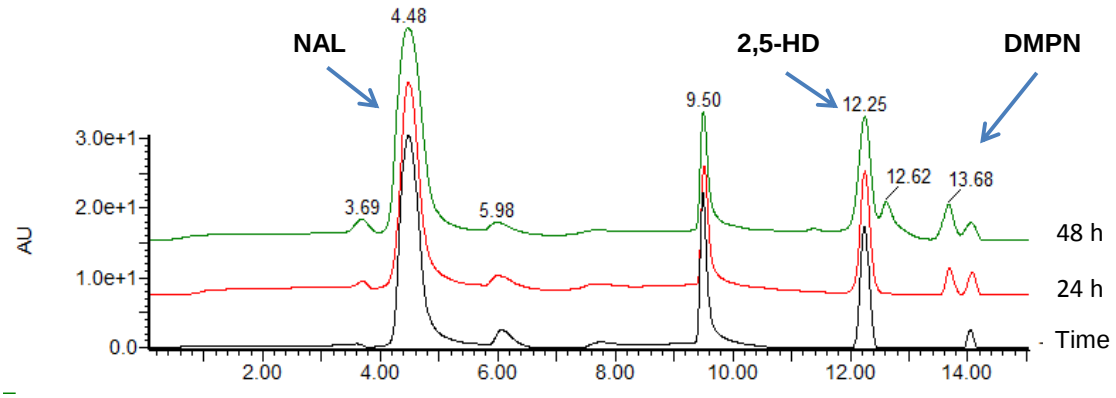


Figure 3.9 Chromatograms obtained by LC with Diode Array detection (210 to 600 nm) for the synthesis of compound DMPN: (—) 24 h and (—) 48 h of incubation reaction. The blak line corresponds to incubation reaccion time, for t=0 min.

It can be seen in the resulting chromatograms the increase in the peak area of the compound as the time of reaction increases, so the incubation time selected for the DMPN synthesis was 48 hours.

DMPN NAC

At the studied conditions, the compound DMPN NAC is detected at Rt=11.1 min as can be seen in Fig. 3.10.

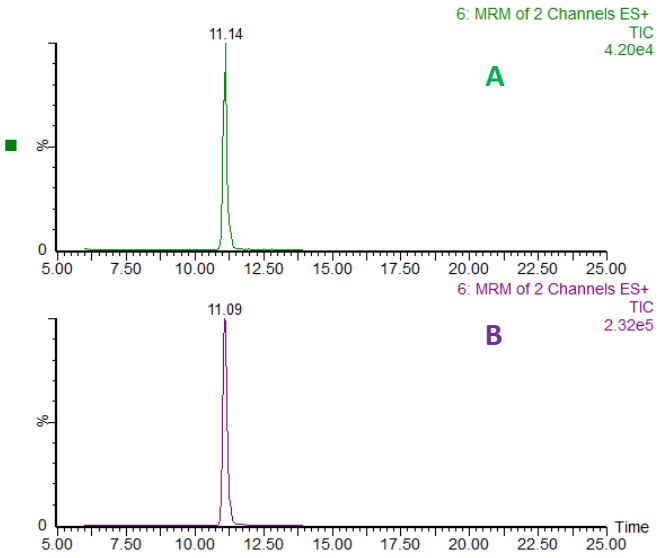


Figure 3.10 Chromatograms obtained in MRM mode, for the compound DMPN NAC: (A) 24 h and (B) 48 h of incubation reaction.

As for DMPN, the formation of the DMPN NAC compound was followed at the beginning and monitored at 2 different reaction times by Diode Array (Fig. 3.11).

As can be seen in the chromatograms, the peak area of DMPN NAC increases with the time of reaction (Fig. 3.11), and the incubation time selected for the compound synthesis was 48 hours.

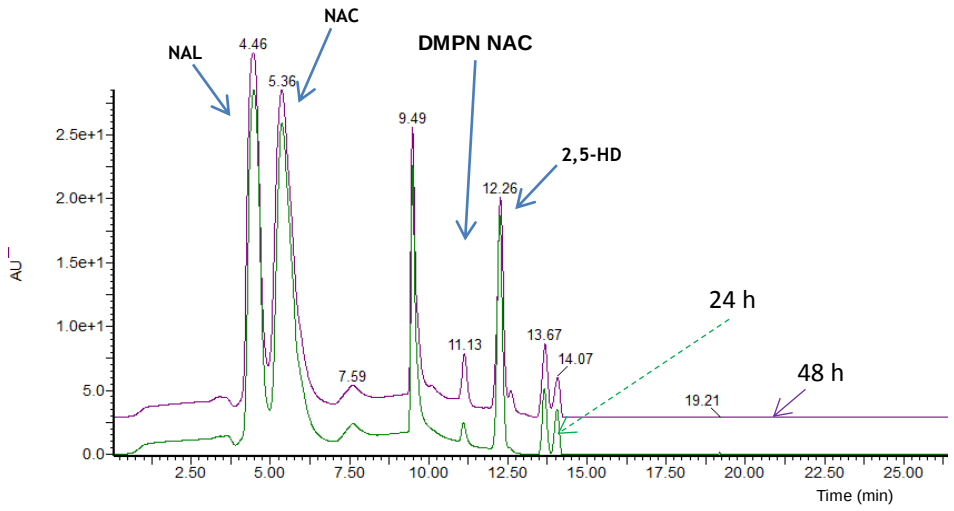


Figure 3.11 Chromatograms obtained by LC with Diode Array detection (210 to 600 nm), for compound DMPN NAC. (—) 24 h and (—) 48 h of incubation reaction.

DMPN GSH

Using the optimized conditions and the transitions chosen for the compound, we confirmed that DMPN GSH is detected at Rt=10,6 min, (Fig. 3.12).

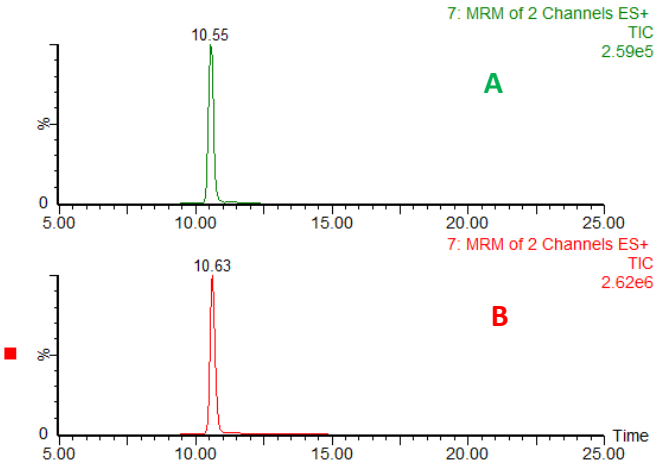


Figure 3.12 Chromatograms obtained in MRM mode for the pyrrole compound synthetized, DMPN GSH: (A) 24 hours and (B) 48 hours of incubation reaction.

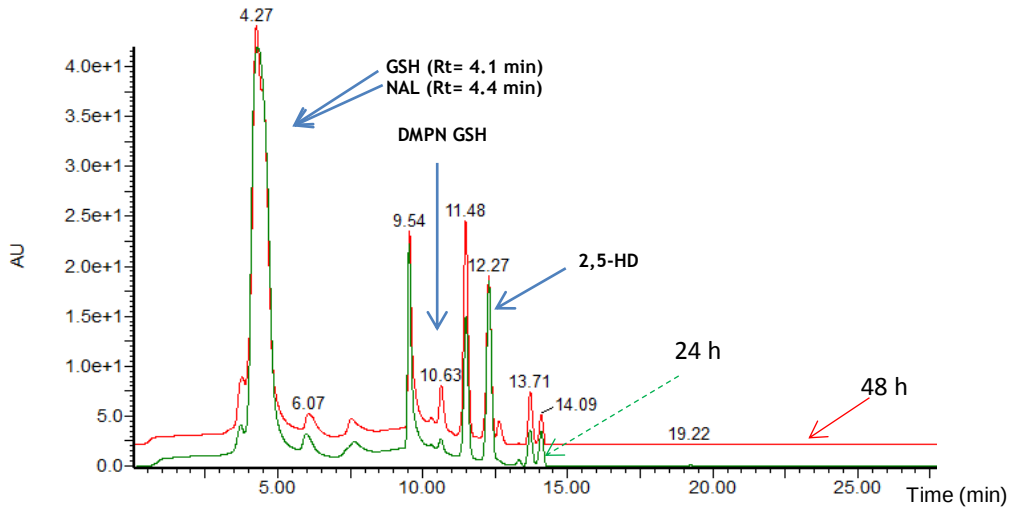


Figure 3.13 Chromatograms obtained by LC with Diode Array detection (210 to 600 nm), for the synthesis of compound DMPN GSH: (—) 24 h and (—) 48 h of incubation reaction.

The formation of DMPN GSH was monitored using Diode Array, at two different reaction times and it was observed that the GSH conjugate peak area increases with time of reaction (Fig. 3.13).

The following figure (Fig. 3.14) shows the chromatograms of each reaction obtained with LC and Diode Array detection (210 to 600 nm), at the incubation time period selected for the study of all synthesized compounds.

The chromatograms of the incubation reactions at 48 h presented here correspond to reactions in aqueous media under N_2, for the three compounds: for DMPN with Rt at ~13.7 min, DMPN NAC with Rt at ~11.1 min and DMPN GSH with Rt at ~10.6 minutes.

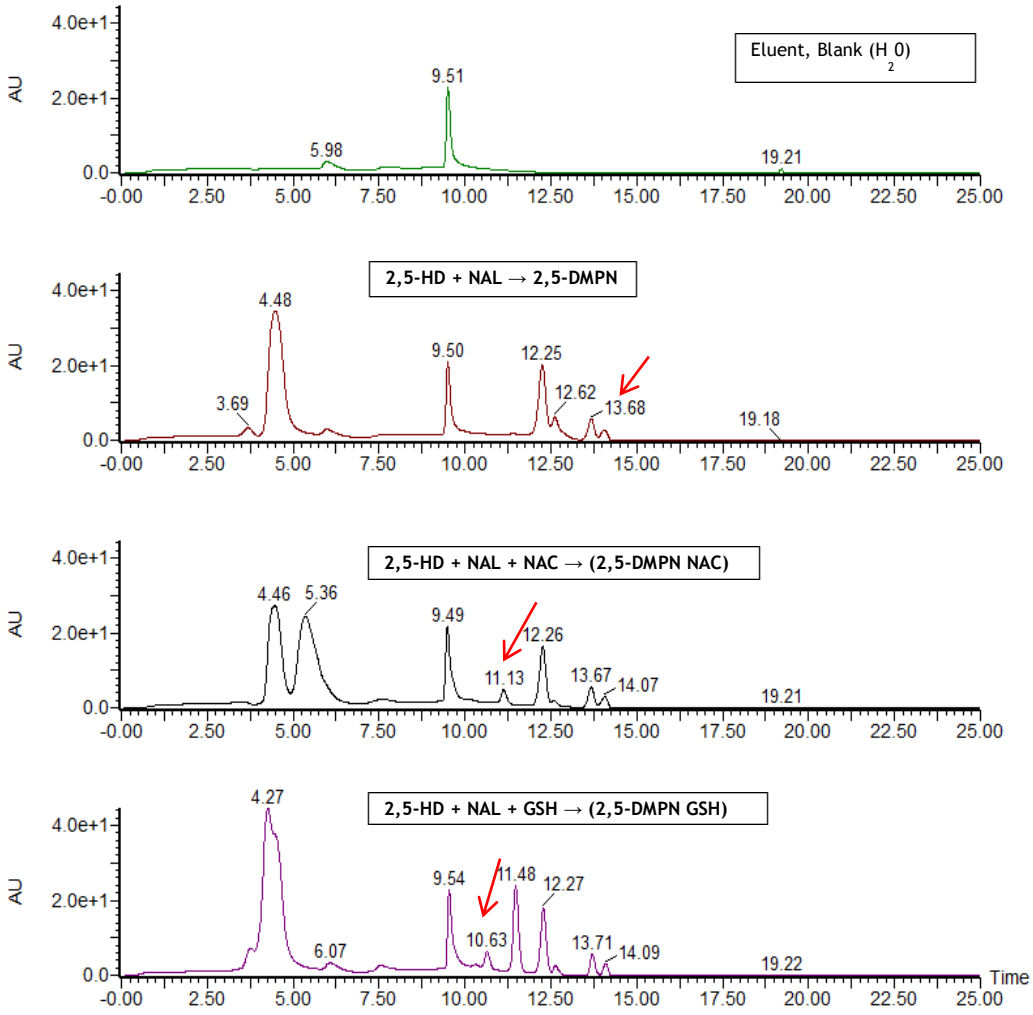


Figure 3.14 Chromatograms obtained by LC with Diode Array Detection (210 to 600 nm) for the pyrrole compounds synthetized: DMPN, DMPN NAC and DMPN GSH, at the standard incubation conditions (37°C, N_2 atmosphere, in the dark) at 48 hours of incubation. (Peaks at Rt= 9.51 min and Rt=5.98 min are presented in the blank).

3.3.3 IDENTIFICATION OF PYRROLE COMPOUNDS IN URINE OF RATS EXPOSED TO 2,5-HD

The chromatogram obtained by MRM mode using the analytical conditions previously described, allowed us to identify for the first time the following pyrrole compounds in urine of exposed rats: DMPN, DMPN NAC and DMPN GSH.

In Figure 3.15 is presented the chromatogram profile corresponding to DMPN, DMPN NAC and DMPN GSH detected in urine of rats exposed to 2,5-HD. DMPN GSH, exhibit a Rt of 10.7 min, DMPN NAC exhibit a Rt of 11.1 min and DMPN a Rt of 13.7 min. Peak area of DPMN is much higher than the peak area of DMPN NAC and DMPN GSH.

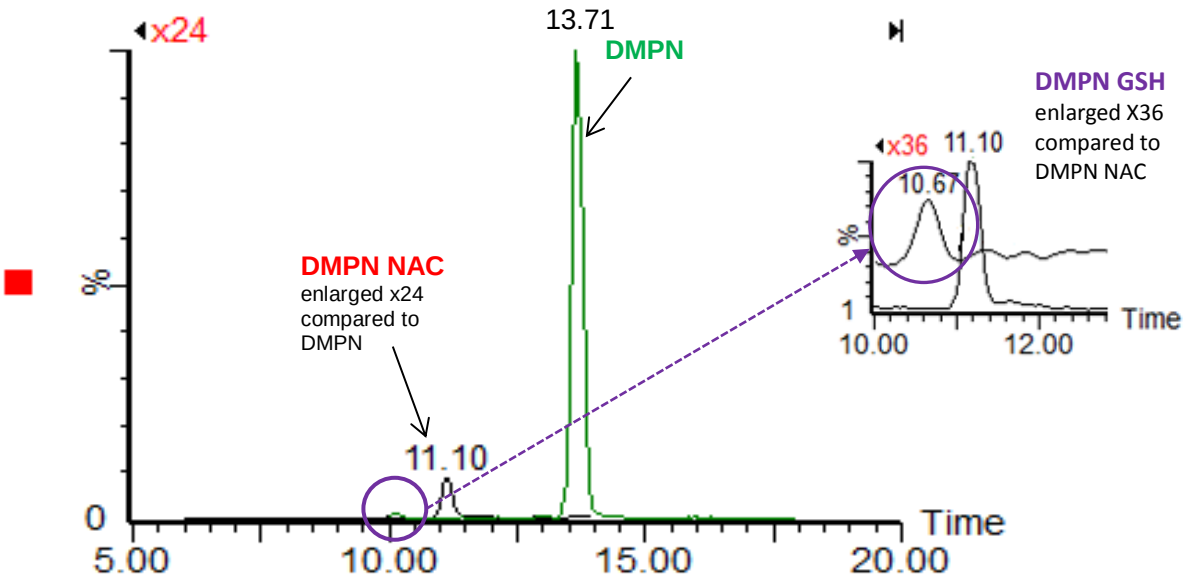


Figure 3.15 Chromatogram obtained in MRM mode of urine of rat exposed to 3 doses of 2,5-HD (400 mg/kg bw) for derived pyrrole compound DMPN GSH (Rt=10.6 min), DMPN NAC (Rt=11.1 min) and DMPN (Rt=13.7 min).

In order to confirm that the compounds detected in urine of the exposed animals corresponded to the synthesized ones and to evaluate the specificity of the method, *MRM*1/*MRM*2 transitions ratio were determined for each derived compound pyrroles in standard solutions and in urine samples.

Figure 3.16 shows the two most important transitions for pyrrole compound DMPN, existing in the aforementioned urine: *MRM*1, 267>162 (quantification) and *MRM*2, 267>225 (confirmation) transitions, as well as the value of their intensities ($5.35x10^6$ and $2.72x10^6$ respectively).

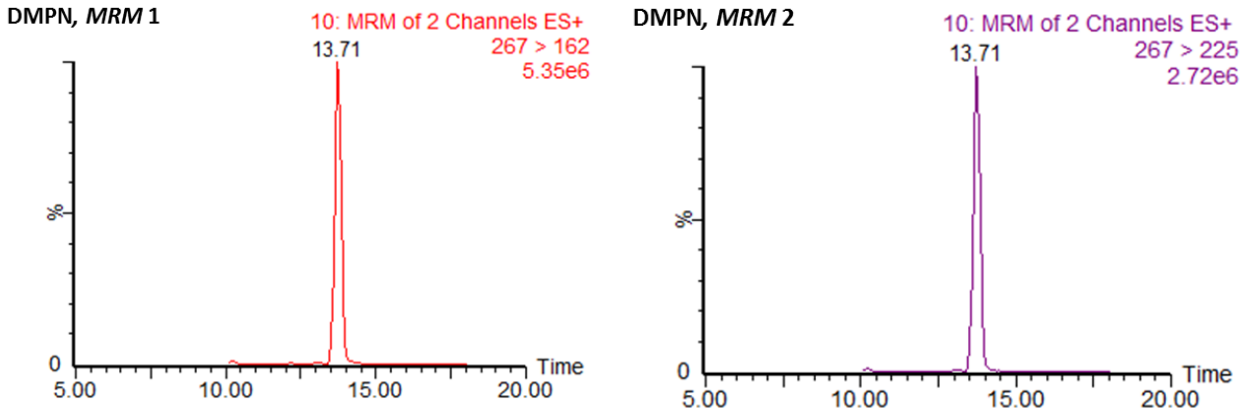


Figure 3.16 Chromatograms in MRM mode of the two most important transitions for pyrrole compound DMPN detected in urine of one rat exposed to 3 doses of 2,5-HD.

In Figure 3.17 are presented the two most important transitions for DMPN NAC, found in urine of one rat exposed to 2,5-HD (3 doses): *MRM*1, 428>299 (quantification) and *MRM*2, 428>410 (confirmation) transitions, as well as the value of their intensities ($1.85x10^4$ and $7.28x10^3$ respectively).

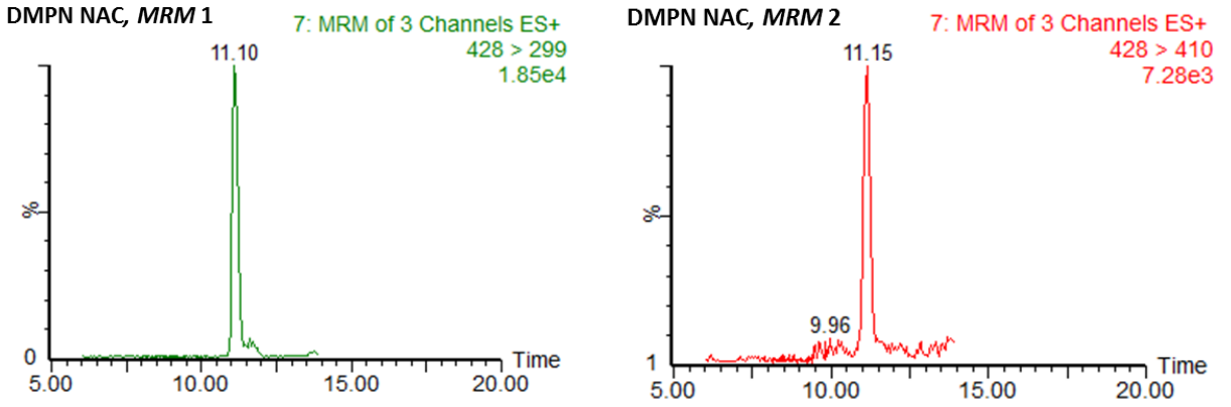


Figure 3.17 Chromatograms in MRM mode of the two most important transitions for derived pyrrole compound DMPN NAC detected in urine of one rat exposed to 2,5-HD (3 doses).

According to the criteria of Commission Decision (2002/657/EC) this value should not be outside the range of RSD ± 20%, when comparing results obtained for samples with the ones obtained for standards.

In this work *MRM*1 transition was used for quantification and *MRM*2 for confirmation. The use of two transitions gives 4 identification points, corresponding to one precursor (1 point) and two product ions (3 points). This parameter fulfils the European Commission requirements for specificity (at least 3 identification points) for confirmation of target compounds.

Table 3.3 Average *MRM*1/*MRM*2 ratio and relative standard deviation for each compound detected in urine of exposed rat.

Compound	Ratio *MRM*1/*MRM*2 Samples (n=21; RSD)
DMPN	2.34 (4.1%)
DMPN NAC	2.25 (4.9%)
DMPN GSH	2.18 (18.1%)
2,5-DMP	3.46 (8.6%)

For each target pyrrole compound, RSD values of *MRM*1/*MRM*2 were lower than 20%, as shown in Table 3.3. Only DMPN GSH presents a value close to 20% for the RSD, due to the fact that the detection of this peak was near the LOD, though the variation was higher. From the results it was possible to conclude that the *MRM* ratios for the peaks detected in the urines can be attributed to the compounds studied DMPN, DMPN NAC, DMPN GSH and 2,5-DMP as these values are according to the criteria previously established.
After identification of pyrrole compounds in rat urine, we proceeded to their quantification.

3.3.4 QUANTIFICATION OF PYRROLE COMPOUNDS IN RAT URINE

Due to the infeasibility of either obtaining commercial standards of pyrrole adducts or purifying those biosynthesized by rats exposed to 2,5-HD, a semi-quantitative approach, based on the use of structurally similar compounds as standards (2,5-DMP structure, Fig. 3.1), has been developed (Manini et al., 2004). When eluted with the same mobile phase composition, pyrrole derivatives, DMPN, DMPN NAC and DMPN GSH, showed similar ESI-MS/MS responses.

Therefore, in order to quantify the data obtained from the identified pyrroles it was assumed that the responses for these compounds are independent of the conjugated part of 2,5-DMP. On this basis a calibration plot for 2,5-DMP was used to estimate the concentration of other identified pyrroles (Manini et al., 1998, 1999, 2004).

From the S/N ratio of the respective MRM chromatograms, as derived from the analysis of standards, it was estimated the limit of detection and quantitation, LOD=17.5 nmol/mL and LOQ=52.6 nmol/mL for 2,5-DMP. The quantification of pyrrole compounds was expressed in terms of the concentration for 2,5-DMP. Under the optimized conditions, the calibration curve of 2,5-DMP was linear in the ranges of 5–100 mg/L. Squared Correlation Coefficients were 0.999 and 0.9993 with water and with control urine, respectively (Fig 3.18).

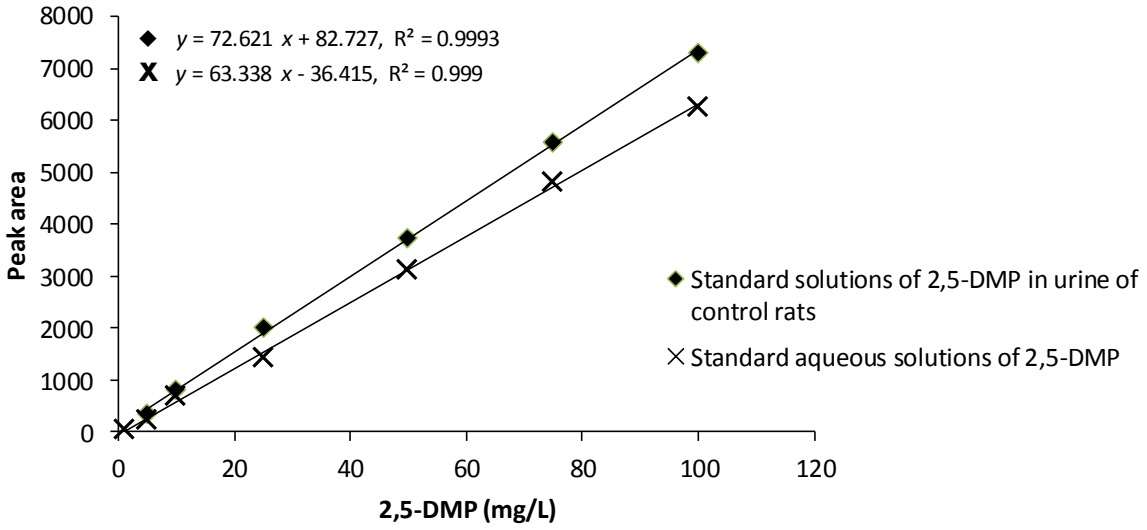


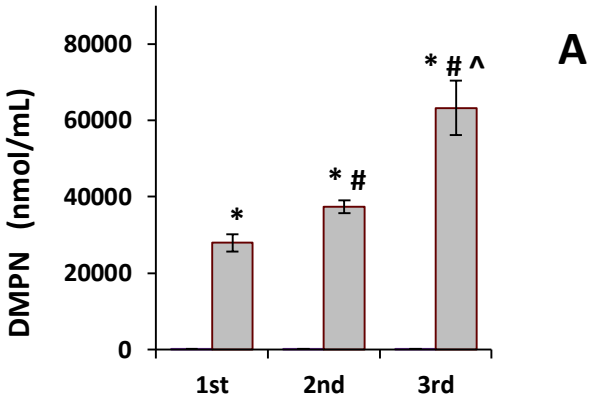
Figure 3.18 Calibration curves for 2,5-DMP standard solutions in the concentrations range 5-100 mg/L.

The repeatability of the method at LOQ is below 10% (RSD=3.4%). The value obtained for matrix effect of 2,5-DMP in urine solutions was 15%. This value indicates that there is a high ion enhancement. Large differences in matrix effect were observed between different bio-fluids (urine, oral fluid, and plasma) (Dams et al., 2003) and between different sample preparation techniques, including direct injection, (Bonfiglio et al., 1999; King et al., 2000; Muller et al., 2002; Dams et al., 2003; Mallet et al., 2004). Although the mechanism and the origin of the matrix effect are not fully understood, signal suppression/enhancement is believed to result from

competition between matrix components and analyte ions in the sprayed solution for access to droplet surface for gas-phase emission (Constantopoulos et al., 1999; Annesley, 2003).

Depending on the environment in which ionization and ion-evaporation take place, this competition may effectively increase (ion enhancement) the efficiency of formation of the desired analyte ion (Bonfiglio et al., 1999; Mallet et al., 2004).
Several approaches have been proposed to compensate for such effect, such as the application of isotopically labeled internal standards (when available at the adequate isotopic purity) rather than analogue internal standards (ISs) (Matuszewski et al., 1998; Jemal et al., 2003), or the application of the standard addition method (Ito and Tsukada, 2002).
According to Manini et al., 2004, one of the main advantages of LC–MS is the compatibility with aqueous matrices and the possibility to minimize or even eliminate sample preparation prior to analysis. However, one limitation is its susceptibility to matrix effect.

Thereby, the results obtained for DMPN and DMPN NAC, after the quantification, are shown in Figure 3.19.
This figure shows (A and B) shows a significant increase (p<0.01) of urinary DMPN and DMPN NAC as compared with control. The increase of DMPN levels is significant (p<0.01) over the three doses (Fig. 3.19 A) while the increase of DMPN NAC levels is not significant between the 2nd and 3rd dose (Fig. 3.19 B).



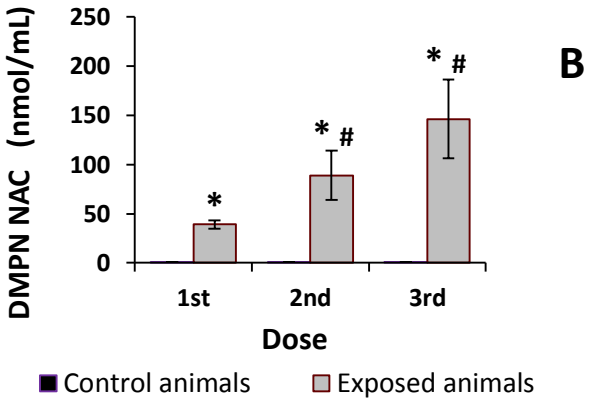


Figure 3.19 Urinary concentration of DMPN (A) and DMPN NAC (B) expressed in nmol/mL, of control rats and rats exposed to 2,5-HD (400 mg/kg bw/48 h). Data represent mean ± SD (n=7). All groups were compared (by Mann–Whitney tests). (*) $p < 0.01$ for exposed animals *vs*. control; (#) $p<0.01$ for 1st dose *vs*. 2nd and 3rd dose and (^) $p < 0.01$ for 2nd dose *vs*. 3rd dose.

In Figure 3.20 it is observed that both compounds have a dose dependent increase on their urinary levels and a linear correlation was established for DMPN (R^2=0.9687) and for DMPN NAC (R^2=0.9915).

After the 3rd administrated dose, the urinary levels of these two compounds are approximately twice (for DMPN), and three times (for DMPN NAC) higher, than their levels after the 1st dose.

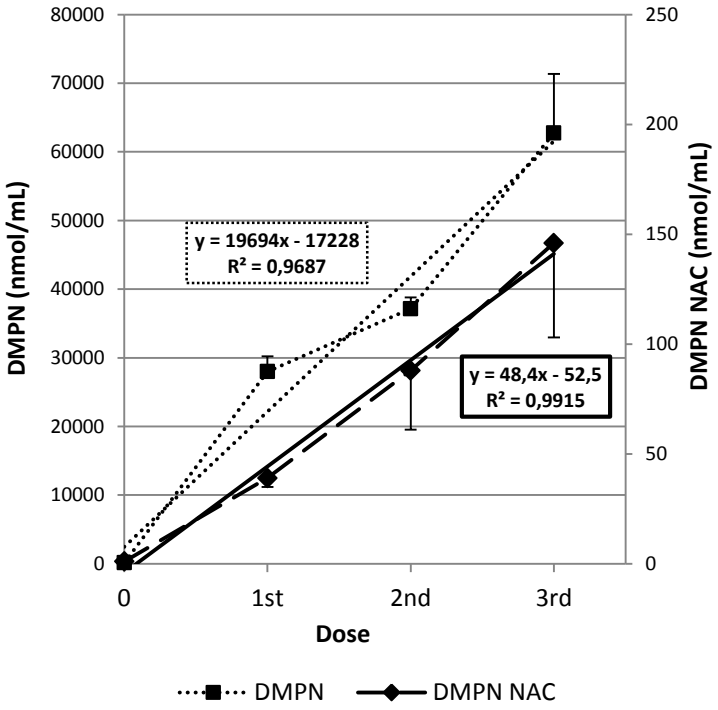


Figure 3.20 Comparison of urinary excretion of DMPN (■) and DMPN NAC (♦) in rats exposed to 3 (ip) doses of 2,5-HD (400 mg/kg bw/48 h). Each point is the (mean + SD) for DMPN, and (mean − SD) for DMPN NAC, for n=7.

The other identified pyrroles, DMPN GSH and 2,5-DMP were detected, however their values were below LOQ.

3.5 DISCUSSION AND CONCLUSIONS

This work identifies for the first time DMPN and its thiol conjugates DMPN NAC and DMPN GSH in urine of rats exposed to 2,5-HD using LC-MS/MS for their characterization.

To achieve this goal, the first task was the synthesis and identification of DMPN and its thiol conjugates, to be used as standards in the identification of the target pyrroles in urine. In figures 3.4, 3.5, and 3.6 it is shown the mass spectra of each synthesized compound after the optimization of instrumental conditions.

The identification of pyrrole compounds in urine of all exposed rats was performed and based in two criteria: Rt and (*MRM*1/*MRM*2) ratio. Fig. 3.15 shows that the Rt of the more concentrated

target analytes (DMPN and DMPN NAC) in rat urine are in accordance with the Rt of the corresponding standards (Table 3.2). On the other hand, in Fig. 3.16 and Fig. 3.17 the two transitions, *MRM*1 and *MRM*2 (of quantification and confirmation), for the pyrroles, DMPN and DMPN NAC are shown.
On comparing, the RSD of *MRM*1/*MRM*2 ratio of target analytes (Table 3.3), with the RSD of the corresponding standards (Table 3.2), a RSD less than 20% was found for all the calculated ratios. All together, the obtained results are in accordance with the requirements of the commission decision (Commission Decision 2002/657/EC) to identify a compound by LC-MS/MS, thus, allowing us to identify, DMPN, DMPN NAC and DMPN GSH in rat urine.

We suggest that the source of DMPN is due to the formation of primary (pyrrolylated N-acetylysine) amino-pyrrole adducts and the source of Cys conjugates is due to the formation of secondary (pyrrolylated cysteine conjugates) thiol-pyrrole adducts. These two types of adducts may occur in most proteins. Our results confirm that the amino-pyrrole adducts are formed in much higher amounts which may be explained by the low level of free thiols present in most proteins limiting the relevance of thiol-to-pyrrole adducts, nevertheless the excretion of this thiol-pyrrole conjugated could be quantified in rat urine and may have a role in 2,5-HD detoxification process.
Concerning DMPN GSH conjugate and 2,5-DMP these pyrroles were detected but their levels were below the LOQ (52.6 nmol/mL).

The comparison of DMPN and DMPN NAC excretion (Fig. 3.19 A and B, and Fig 3.20) evidences (i) the high urinary DMPN excretion as compared with urinary DMPN NAC, (ii) the linear correlation between the DMPN or DMPN NAC levels at the administrated doses.

In conclusion, DMPN is a strong candidate to be proposed as a new biomarker of exposure to 2,5-HD and research is needed to study its role as a potential biomarker of neurotoxic effect.

CHAPTER 4

ROLE OF NAC IN PROTECTING AGAINST 2,5-HEXANEDIONE NEUROTOXICITY IN A RAT MODEL

4.1 INTRODUCTION

Risk prevention against n-hexane neurotoxicity is a relevant issue towards the measures to be proposed in occupational toxicology. One of the approaches to be applied in protection against n-hexane neurotoxicity is the development of antagonists that could reduce or eliminate its toxicity.

It is well known that 2,5-HD is the metabolite responsible by n-hexane neurotoxicity and urinary levels of total 2,5-HD (free 2,5-HD + 4,5-dihydroxi-2-hexanone) have been used in routine analysis as a biomarker of occupational exposure to n-hexane (Perbellini et al., 1990a,b; Biological Exposure Values for Occupational Toxicants and Carcinogens, 1994). However, in 2001, the ACGIH recommended the determination of "free" instead of "total" 2,5-HD, since "free" 2,5-HD, indicates the amount of 2,5-HD that escapes to the detoxification process, and probably is a better predictor of the neurotoxic risk than the conjugated metabolites that are rapidly excreted in urine (Manini et al., 1999, 2004; Mateus et al., 2001). On the other hand, this γ-diketone, may bind to amino groups of lysine in axonal proteins forming 2,5-DMP (2,5-dimethylpyrrole) primary adducts or binds to free thiols in proteins forming secondary pyrrole adducts (Zhu et al., 1994, 1995, 1997; Torres et al., 2010; Torres et al., 2012a, 2012b; Mateus et al., 2012). However, the low levels of free thiols present in most proteins limit the relevance of thiol-to-pyrrole adducts.

Several authors (Graham et al., 1995; Costa, 1996) have proposed the determination of Pyrrole Like Substances (PLS) as a biomarker of cumulative exposure to n-hexane, as it reflects the dose of free 2,5-HD that escapes detoxification and reaches its target proteins. The PLS have been detected in hen CNS tissues (DeCaprio et al., 1983), rat hair (Johnson et al., 1995; Lack et al., 2002), rat and hen blood (DeCaprio et al., 1982, 1988; Graham et al., 1995) and rat and human urine (Kessler et al., 1990; Santos and Batoréu, 1991, 1993). To detect the PLS a simple and sensitive colorimetric assay with *p*-DMBA (Erhlich's reagent) was used and was established (Kessler et al., 1990) a correlation between PLS and 2,5-HD urinary levels. However, this method does not identify the different pyrrole compounds excreted in urine, hindering the meaning of pyrroles excretion in several neurophysiologic conditions (Hidalgo et al., 1998) and in different exposure situations (Lamé et al., 1990).

In Chapter 3, we described the synthesis and identification of pyrrole compounds by ESI-LC-MS, and DMPN and DMPN NAC were also quantified in urine of rats exposed to 2,5-HD

(Torres et al., 2014). At this point, it was imperative to investigate the toxicological meaning of excretion of the identified pyrroles.

4.2 NAC AS A POTENTIAL CHEMOPREVENTIVE AGENT

The role of NAC in protecting CNS was investigated by Banaclocha (2001), proposing its potential mechanism of action based on the assumption that NAC can cross the blood-brain barrier, and exert its beneficial effects at the level of CNS (Drigen, 2000).
It is well known that NAC is a precursor of Cys and an excellent source of sulfhydryl (-SH) groups that restore the reduced cellular glutathione (GSH) and increases its pool (Zafarullah et al., 2003; Chen et al., 2008; Moschou et al., 2008; Circu and Aw, 2010). GSH plays a central physiological role in maintaining the body homeostasis and in protecting cells against oxidant toxicants, of either exogenous or endogenous source. Unfortunately, the large GSH molecule is not transported efficiently into cells. Furthermore, Cys, which is the rate-limiting amino acid in the intracellular synthesis of this tripeptide, is toxic to humans (De Flora et al., 2001). Thus, to obtain an increased amount of cellular GSH levels we selected NAC. It is an amino acid derivative with low toxicity which readily deacetylated in cells to yield Cys (Bridgeman et al., 1991) and promotes intracellular glutathione (GSH) synthesis. Besides this activity as a GSH precursor, NAC is, *per se*, responsible for protective effects in the extracellular environment, mainly due to its nucleophilic and antioxidant properties, which influence the toxicokinetics of several xenobiotics (Schulz et al., 2000; Aoyama et al., 2008).

NAC is an important antioxidant and a free radical-scavenging agent that increases intracellular GSH (Griffith and Meister, 1979), a major component of the pathways by which cells are protected from oxidative stress (Meister, 1988; De Flora et al., 2001; Arakawa et al., 2006). The efficacy of NAC in protecting cells from apoptosis has generally been interpreted within the context of a mechanism involving oxidative stress (Ferrari et al., 1995; Arakawa et al., 2006). NAC, at a concentration of 1 mM, became an excellent neuroprotective compound, causing almost 100% neuroprotection against metal-induced neurotoxicity which is mainly caused by the increase of reactive oxygen species (ROS) (Moschou et al., 2008).

It has been shown to exert survival promoting actions in several cell systems (Banaclocha et al., 1997) and fluorescence studies revealed that neuronal death was suppressed by NAC (Ferrari et al., 1995). Cys is transported mainly by alanine-serine-cysteine (ASC) system, a ubiquitous system of Na^+-dependent neutral amino acid transport, in a variety of cells (Arakawa et al., 2006). However, NAC is a membrane-permeable Cys precursor that does not require active transport and delivers Cyst to the cell in this own unique ways (Sen, 1998; Aoyama et al., 2006).

The aim of this work was to study the interference of NAC on 2,5-HD neurotoxicity and to investigate the toxicological meaning of urinary aminoderived pyrroles and thiol-pyrrole conjugates excretion.
To achieve these objectives we analyzed urinary 2,5-HD, 2,5-DMP, DMPN, DMPN NAC and DMPN GSH levels, and performed behavioral assays in rats exposed to 2,5-HD and co-exposed to 2,5-HD and NAC.

4.3 MATERIAL AND METHODS

4.3.1 CHEMICALS

2,5-Hexanedione (2,5-HD, 99%) was purchased from Fluka, 2,5-dimethylpyrrole (2,5-DMP, 98%), Nα-acetyl-L-cysteine (NAC, 99%), Nα-acetyl-L-lysine (NAL, 98%), γ-L-glutamyl-L-cysteinyl-glycine (GSH, 98%) and cyclohexanone (99,8%) were obtained from Sigma–Aldrich, 4-dimethylaminobenzaldheyde (DMAB, 99%), boron trifluoride–methanol complex, ammonium acetate, acetonitrile *Lichrosolv* and ethanol *p.a*. were from Merck. Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

4.3.2 INSTRUMENTATION

4.3.2.1 Gas Chromatography and Mass Spectrometry Method

Quantification of 2,5-HD in urine was performed by GC-MS, Perkin Elmer, Clarus 500 Gas Chromatograph, Claurus 560D Mass Spectrometer, with a quadrupole detector in selected ion monitoring (SIM) mode and a DB-17 capillary column (30 m x 0,53 mm, 1µm).
For GC-MS detection, an electron ionization system was operated in electronic impact (EI) mode with ionization energy of 70 eV, Helium gas (99.999%) was the carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 µL was employed. The injector temperature was maintained at 225 °C, the ion-source temperature was 200 °C, and oven temperature was programmed from 70 °C (1 min), with an increase of 15 °C/min to 150°C (20 min).
The mass-detector was the Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass, version 5.2.
Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST MS Search, version 2.0).

4.3.2.2 Liquid Chromatography and Mass Spectrometry Method

Urine was analyzed by ESI-LC-MS/MS, for pyrrole compounds determination. A Waters Alliance 2695 (Waters®, Ireland) LC system equipped with a quaternary pump, solvent degasser, auto sampler, column oven and a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) with an electrospray ionization source (ESI), already mentioned in Chapter 3.

4.3.3 ANIMALS

Male Wistar rats (223 ± 22 g) from Charles River Laboratories® (Barcelona), were housed at controlled temperature, humidity and a 12-h light/dark cycle. A preliminary week acclimatization period, and during the exposure, the general conditions of the animals were checked daily. Animals had free access to water and rat standard food (Letica Ref. IPM-R20).

All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

4.3.4 EXPOSURE OF RATS AND URINE COLLECTION

The selection of dose, time and frequency of exposure was based on previous experiments performed in our lab. (Mateus et al., 2001, 2002; Torres et al., 2010, 2012a, 2012b).

Concerning the selection of NAC doses, it is reported that has a low toxicity in experimental animals, being its oral LD_{50} > 10 g/kg body weight in both rats and mice, and the LD_{50} after intravenous administration 4.6 g/kg in mice and 2.8 g/kg in rats (De Flora et al., 2001).

After the acclimatization period, the animals were randomly assigned to four groups (n=7): Group I – 400 mg 2,5-HD/kg bw/48-h (ip); Group II – 400 mg 2,5-HD/kg bw/48-h (ip) + 200 mg NAC/kg bw/day (drinking water); Group III – 200 mg NAC/kg bw/day (drinking water); Group IV – saline solution (ip). The Group II was pre-exposed to NAC (200 mg/kg bw) in drinking water, one week before the co-exposure to 2,5-HD+NAC. The Group III was also pre-exposed, (see Table 4.1).

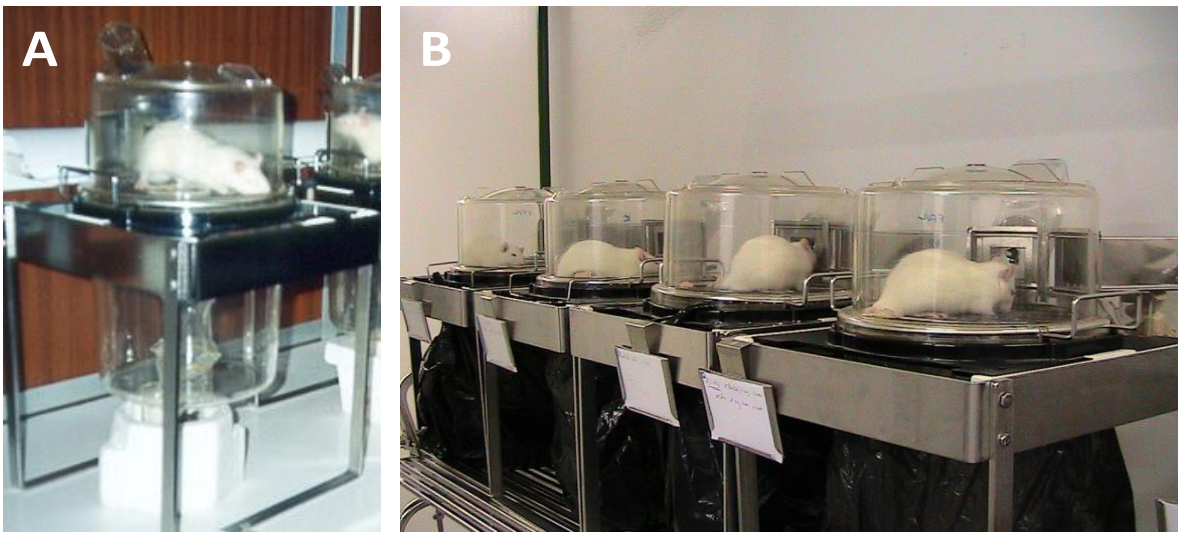


Figure 4.1 Collection of 24-hour urine from rats kept in metabolic cages. **A**: metabolic cages provided with urine-feces separator. **B**: the collection of urine should be done protected from light to prevent degradation of pyrrole compounds.

During the 24 hours urine collection, animals were kept in metabolic cages provided with urine-feces separator (Fig. 4.1). All urine samples were collected over ice and out of light, and then were centrifuged to remove particulate matter. Urine samples were stored at –80ºC until analysis.

2,5-Hexanedione was dissolved in a sterile saline solution and was ip administered to the animals with a 48-hour interval. NAC was added to the drinkable water supplied to the animals. NAC solution concentration was calculated considering an average rat's water intake, between 10 and 20 mL (Ecobichon, 1997). In Table 4.1 is presented the experimental design of animal study.

Table 4.1 Animal assay protocol.

Group	Treatment solution[a]	Doses
HD (**I**)	400 mg 2,5-HD/kg bw/48-h	4 (HD)
(HD + NAC) (**II**)	400 mg 2,5-HD/kg bw/48-h + 200 mg NAC/kg/day	4 (HD) + 15 (NAC)
NAC (**III**)	200 mg NAC/kg/day	15 (NAC)
Control (**IV**)	Sterile saline/48-h	4 (saline)

(a) All treatment solutions were prepared freshly on a daily basis. 2,5-HD was administered *via* ip injection and NAC was administered *via* oral in drinking water (7 animals/group).

4.3.5 DETERMINATION OF 2,5-HEXANEDIONE

4.3.5.1 Urine Sample Pre-Treatment

Determination of Free 2,5-HD in urine

To 5 mL of centrifuged urine, was added 100 mL of a 10.4 mM cyclohexanone solution in water (Internal Standard) and 1.5 g of NaCl. After addition of 1 mL of dichloromethane, samples were rotated and subsequently centrifuged. Organic phase was collected for analysis on the gas chromatography system.

Determination of Total 2,5-HD in urine

Five mL of urine was hydrolyzed with HCl (pH 0.1) at 100 ºC, and the formed 2,5-HD was extracted and determined by the above procedure (Mateus et al., 2000).

4.3.5.2 Gas Chromatography-Mass Spectrometry Analysis

A 1-µL aliquot of the dichloromethane extract was injected onto the GC system (van Engelen et al., 1995; Mateus et al., 2000).
Individual standard solution (100 ppm) of 2,5-HD, was injected into GC and analysed in the positive ion mode in order to achieve the best conditions of analysis.

4.3.5.3 Validation parameters for GC-MS Method

The calibration curves for quantitative analysis of 2,5-HD were calculated by the analysis of standard solutions. These were performed by spiking pooled control urines with 2,5-HD. They were also used to study the linearity of the method. To control the analytical conditions stability, standard solutions were frequently included during sample analysis.

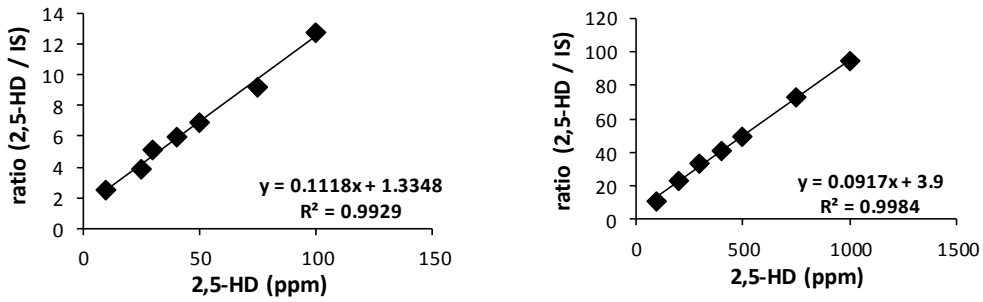


Figure 4.2 Calibration curves for 2,5-HD standard solutions in urine of unexposed rats, obtained by GC-MS for different concentrations. Each value is the average of three determinations.

Under the optimized conditions, the calibration curves of 2,5-HD were linear in the concentration ranges of 10-100 ppm and 100-1000 ppm (Fig. 4.2).

The instrumental limit of detection (LOD) was estimated from the standard deviation of the response and the Slope. It can be expressed as LOD = 3.3 x σ / S, where σ is the standard error of the response B (intercept coefficient) and S is the slope of the calibration curve. The slope S was estimated from the calibration curve of 2,5-HD for concentrations between 10 and 100 ppm. The estimation of σ was also based on the calibration curve. The residual standard error of the regression line or the standard error of y-intercepts (B) of regression line was used as the standard error (ICH-Q2-R1).

The instrumental limit of quantification (LOQ) is 3×LOD (Commission Decision 2002/657/EC; ISO 8466-1, 1990).

By using SPSS SATISTICS® version 21, the following values were obtained for B, S and σ (Table 4.2).

Table 4.2 Results for σ and S.

	Unstandardized Coefficients	
		Std. Error
Ratio (2,5-HD/IS)	1.335 (B)	0.233**(σ)**
2,5-HD (ppm)	0.112 **(S)**	0.004

Through the values of σ and S, it was obtained for 2,5-HD analyzed by GC-MS, the following results: LOD=6.8 mg/L (59.6 nmol/mL) and LOQ=20.6 mg/L (180.4 nmol/mL).

The repeatability of the injection was determined by injecting consecutively, 10 times, the same solution of 2,5-HD (100 mg/L) in urine of unexposed rat. The value obtained had RSD=6.7%.

In summary, the method described above proved to have adequate sensitivity and a good repeatability. Additionally, it was robust to small analytical conditions variation.

4.3.6 DETERMINATION OF THE PYRROLE DERIVATIVES

To confirm the presence of pyrrole compounds, prior to analysis by ESI-LC-MS/MS, we proceeded to a qualitative analysis of each urine sample, by a colorimetric method with Ehrlich's reagent.

Liquid Chromatography and Mass Spectrometry Method

Standard solutions of 2,5-DMP, or the synthesized compounds (DMPN, DMPN cysteine conjugates: DMPN NAC and DMPN GSH) were infused into the mass spectrometer and scanned in the positive ion mode in order to achieve the best conditions of analysis (cone and capillary voltage) corresponding to higher signal/noise ratio to detect the molecular ion, as it was referred in Chapter 3.

The analytical procedure for analyzing pyrrole compounds obtained in this study is identical to that used in the previous chapter.

4.3.7 BEHAVIORAL ASSAYS

The behavioral tests were based on the sequential observation of locomotor animal acts when they were placed in a new environment (reviewed in exploratory activity). Observations took place in the home cage and on an open field arena (Fig. 4.3). During this time the different movements, physical appearance, and reactions to various stimuli were evaluated.

Behavioral tests were repeated with the same procedure throughout the experiment for all animals, to eliminate other factors that could affect the reproducibility of the measured parameters.

The behavioral assays were always performed at the same hour, just before the 2,5-HD ip treatment.

To minimize the observer bias, the observations were performed without knowledge of the group to which the animal belonged (blind observation).

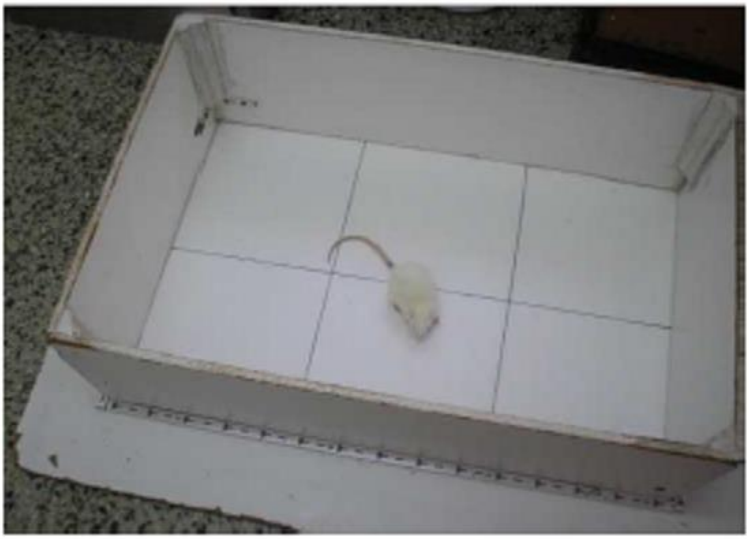


Figure 4.3 Open field test.

The open field was placed in a restricted access room, separated from the rat room. The open field has an area of 90 cm x 60 cm, with walls to limit the field of 35 cm in height and divided into six equal-sized quadrants, as shown in Figure 4.3 (Ladefoged et al., 1994; Moser 1999).

Traits recorded consisted of counting the number of rectangles traversed and the number of times the rats were placed on their hind legs supported or was supported by the side wall of the field. The count took place during the period of five minutes after placing the mouse in the center of the field. This placement was always made at the same place, *i.e.*, in the same quadrant to all rats.

4.4 RESULTS

During the experiment were analysed urinary biomarkers and were performed motor activity assays in all four groups of rats.

The determined urinary biomarkers were:

- Free and total 2,5-HD determined by GC-MS,
- Pyrrole compounds as pyrrole DMPN, DMPN NAC, DMPN GSH and 2,5-DMP determined by ESI-LC-MS/MS

The performed motor activity assays were:

- Rearing and ambulation in open field.

4.4.1 URINARY BIOMARKERS

4.4.1.1 Free and Total 2,5-HD

Identification of 2,5-HD in rat urine

Gas chromatography analysis, allowed us to identify 2,5-HD in urine of all rats (Rt~4.38 min, for 2,5-HD). Total ion chromatograms were performed in selected ion monitoring (SIM) mode. The chromatogram profile corresponding to 2,5-HD determined in urine of one rat exposed to 2,5-HD is presented in Figure 4.4.

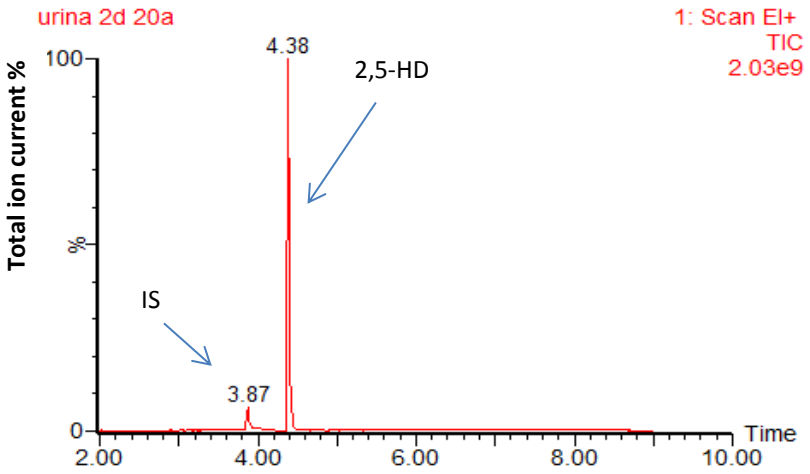


Figure 4.4 Total ion chromatogram obtained in selected ion monitoring mode (SIM) for 2,5-HD and IS, of urine of one rat exposed to 2,5-HD.

In Figure 4.5 is shown the product-ions mass spectrum from molecular ion for 2,5-HD, *m/z* 114, obtained through an urine of an exposed animal to 2,5-HD. Multiple ion detection was used to obtain the molecular ion of 2,5-hexanedione, *m/z* 114, and the fragmentation ions, *m/z* 43, 71 and 99. The ions *m/z* 71, 99 and 114 served as qualifiers to identify 2,5-hexanedione by their abundance relative to the base fragment ion *m/z* 43.

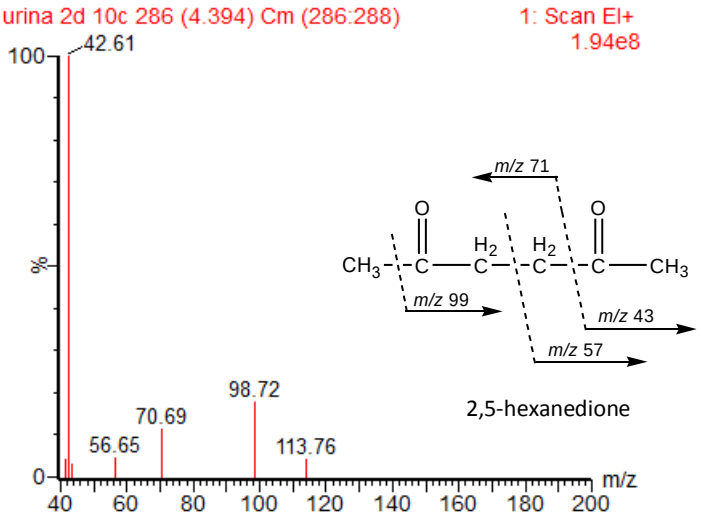


Figure 4.5 Product-ions mass spectrum from molecular ion of 2,5-HD, *m/z* 114, and the fragmentation ions *m/z* 43, 57, 71 and 99. Spectrum obtained through urine of an exposed animal to 2,5-HD.

To confirm the results of fragmentation ions *m/z* 43, 57, 71, 99 and 114 for 2,5-HD and *m/z* 42, 55, 69, 70 and 98 for cyclohexanone (IS), an interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST MS Search, version 2.0).

Quantification of Free and Total 2,5-HD in rat urine

The quantification of free 2,5-HD is presented in Figure 4.6. It is shown a significant decrease ($p< 0.01$) of free 2,5-HD levels in rats co-exposed to 2,5-HD+NAC as compared with its values in rats exposed to 2,5-HD alone. In Figure 4.7 is shown the significantly higher levels ($p<0.01$) of total 2,5-HD in rats co-exposed to 2,5-HD+NAC when compared with the levels in rats exposed to 2,5-HD. The values obtained with control rats (Groups III and IV) are not represented as their values are below the limit of quantification (LOQ=20,6 mg/L).

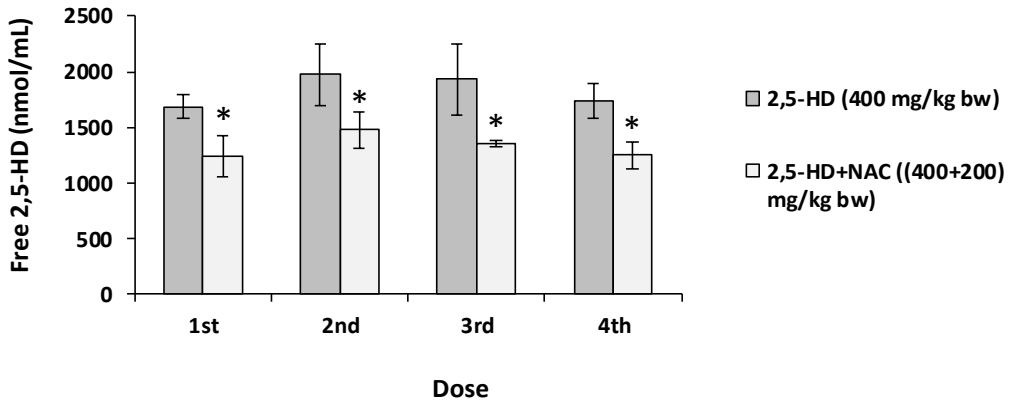


Figure 4.6 Urinary excretion of free 2,5-HD (expressed as nmol/mL) in rats exposed to 2,5-HD (Group I) and co-exposed to 2,5-HD+NAC (Group II). Values represent means ± SD for n=7. (*) $p<0.01$ for Group I *vs.* II. Groups were compared by Mann–Whitney tests.

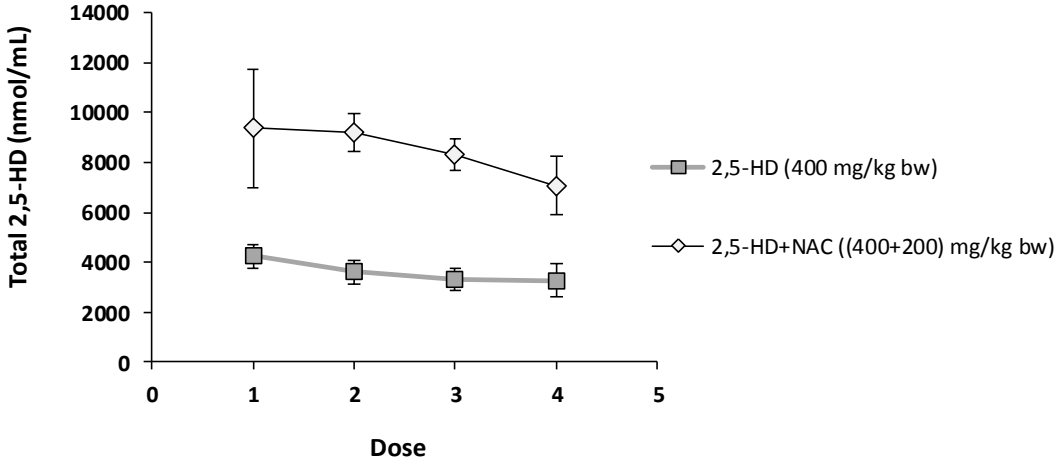


Figure 4.7 Urinary excretion of total 2,5-HD (expressed as nmol/mL) in rats exposed to 2,5-HD (Group I) and co-exposed to 2,5-HD+NAC (Group II). Values represent means ± SD for n=7.

On comparing figures 4.6 e 4.7 it is evident that the higher values of urinary free 2,5-HD are found in Group I, and Group II present the higher values of total 2,5-HD.

4.4.1.2 Pyrrole compounds

Identification of pyrrole compounds in rat urine

Besides, the identification of urinary DMPN and of DMPN NAC already referred in Chapter 3, in this experiment it was possible to identify the other pyrrole cysteine conjugate, DMPN GSH, in urine of rats co-exposed to 2,5-HD+NAC.

The fragmentation from precursor ion *m/z* 572 in fragments *m/z* 443 and *m/z* 294 was obtained through a urine of an exposed animal to 2,5-HD+NAC. In Figure 4.8, it is shown the two most important transitions for derived pyrrole compound DMPN GSH, in urine of rat exposed to 4 doses (2,5-HD+NAC): *MRM*1, 572>297 (quantification) and *MRM*2, 572>443 (confirmation) transitions, as well as the value of their intensities.

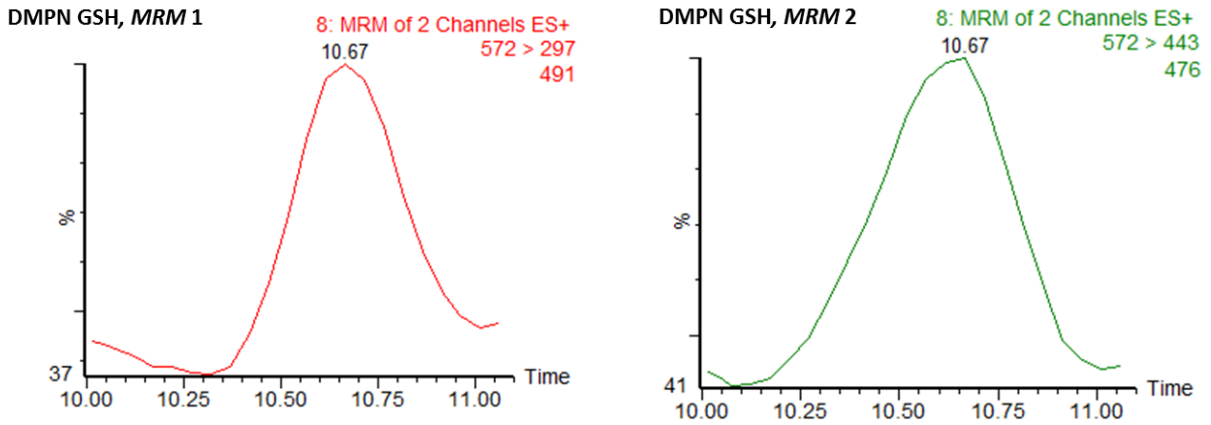


Figure 4.8 Chromatograms in MRM mode of the two most important transitions for derived pyrrole compound DMPN GSH detected in urine of rat exposed to 4 doses (2,5-HD+NAC): *MRM*1, 572>297 (transition of quantification) and *MRM*2, 572>443 (transition of confirmation), as well as the value of their intensities.

Quantification of pyrrole compounds in rat urine

DMPN

In Figure 4.9, are represented several chromatograms in MRM mode of pyrrole compound DMPN detect in urine of all groups of rats (after treatment with 4 doses of 2,5-HD).

In these chromatograms, the rats treated with 2,5-HD (chromatogram in red) have the higher urinary levels of DMPN. It is also evident that the animals co-exposed to 2,5-HD and NAC present lower urinary levels of DMPN, (chromatogram in green). The control groups (III and IV) (chromatograms violet and brown) present values near the detection limit.

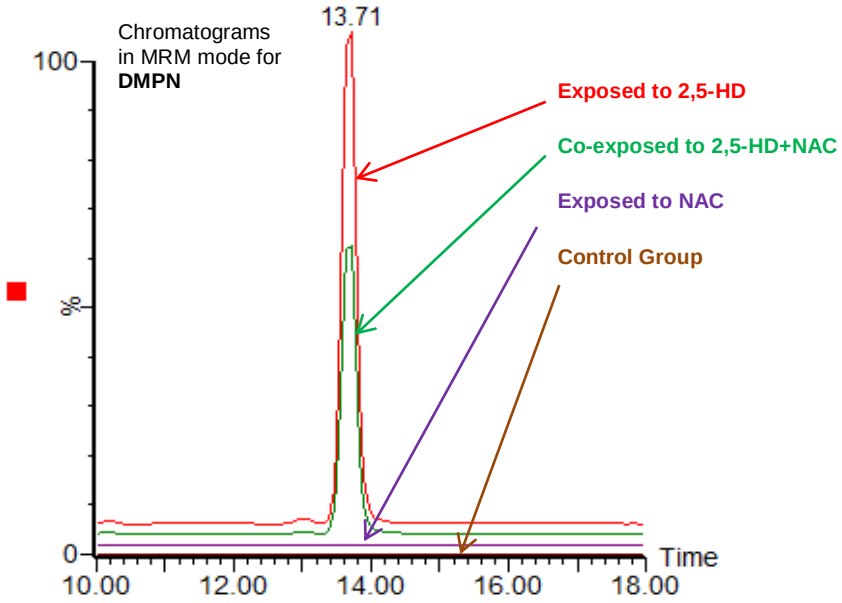


Figure 4.9 Chromatograms obtained in MRM mode for pyrrole compound DMPN, of urine of rats exposed to 4 doses of 2,5-HD (—), co-exposed to 2,5-HD+NAC (—), exposed to NAC (—) and Control (—). (The lag between the baselines of several chromatograms is 2% relative intensity).

Figure 4.10 shows the mean values of urinary levels of DMPN in 24 hours urine animals, for each administrated dose. Levels of urinary DMPN in rats exposed to 2,5-HD are significant (p<0.01) higher compared with its levels in control.

The following figure (4.10) also shows that the urinary levels of DMPN decreases significantly in rats co-exposed to 2,5-HD+NAC as compared with its values in rats exposed to 2,5-HD alone. This difference is noticeable immediately after the 1st dose of co-exposure.

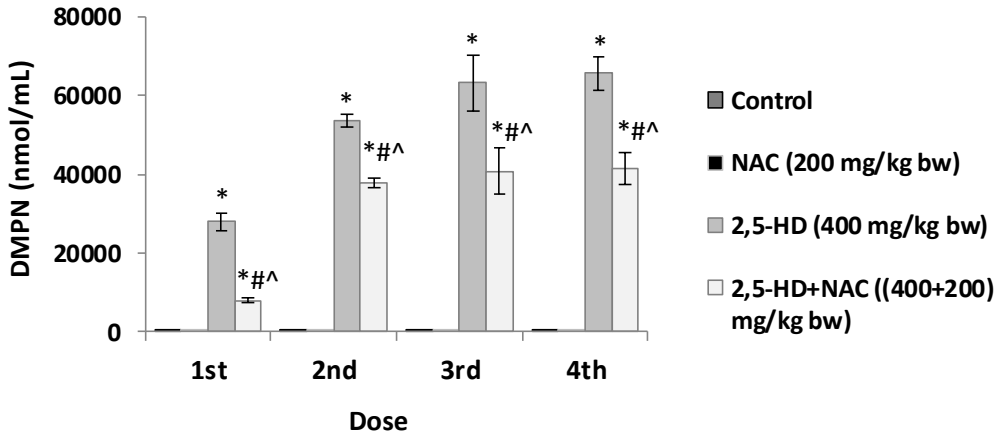


Figure 4.10 Urinary excretion of pyrrole compound DMPN of Group IV, Group III, Group I and II. Data represents the mean ± SD (n=7). Groups were compared by Mann–Whitney tests. (*) $p<0.01$ for Group IV *vs*. I and II; (#) $p<0.01$ for Group III *vs*. Group II; (^) $p<0.01$ for Group I *vs*. Group II.

DMPN NAC

The chromatograms profile corresponding to DMPN NAC detected in urines of rats of: Group I (4 doses 2,5-HD), Group II (4 doses 2,5-HD + 15 doses NAC), Group III and Group IV is presented in Figure 4.11. It can be seen that rats exposed to 2,5-HD show an urinary excretion of DMPN NAC significantly higher than controls (saline treated (IV) and treated with NAC (III)), and significantly lower than those co-exposed to (2,5-HD+NAC).

After the quantification of this pyrrole compound in all urines of Groups I, II, III and IV we represent the graph in Figure 4.12. In this figure it can be seen that rats co-exposed to 2,5-HD+NAC show a significantly ($p<0.01$) higher urinary excretion of DMPN NAC when compared with 2,5-HD exposed rats. This significant difference between the two Groups is noticeable immediately after the 1st dose.

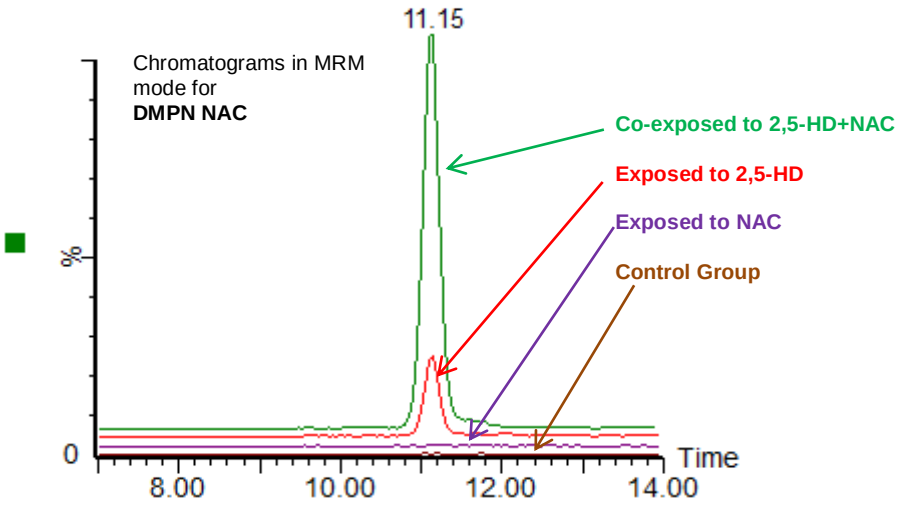


Figure 4.11 Chromatograms obtained in MRM mode for pyrrole compound DMPN NAC, of urine of rats exposed to 4th dose of: 2,5-HD (—), co-exposed to (2,5-HD+NAC) (—), exposed to NAC (—) and Control (—).(The lag between the baselines of several chromatograms is 2% relative intensity).

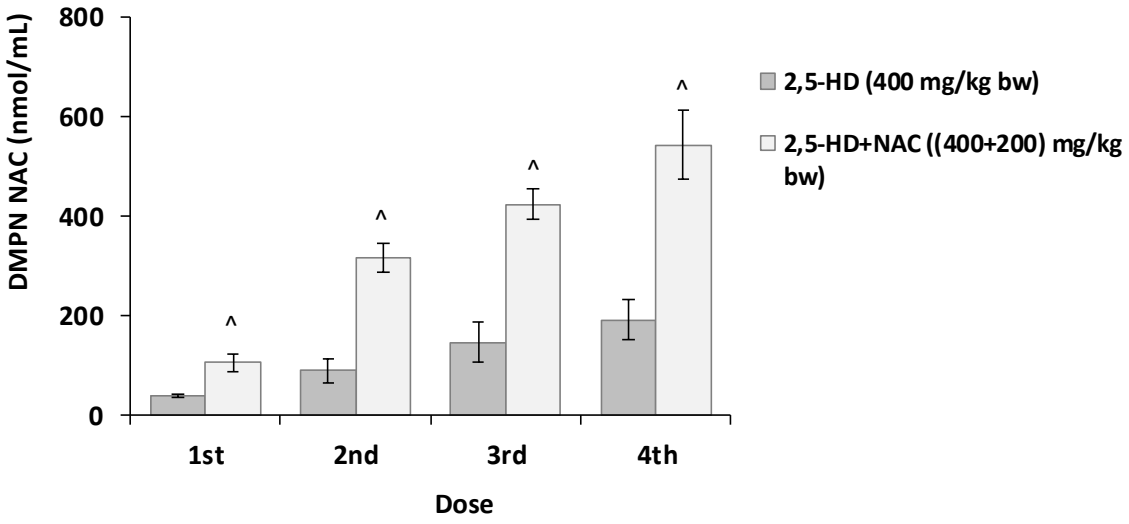


Figure 4.12 Urinary excretion of pyrrole compound DMPN NAC of Group I and II. Data represents the mean ± SD (n=7). (^) $p<0.01$ for Group I *vs.* Group II. Groups were compared by Mann–Whitney tests.

We also try to determine the levels of 2,5-DMP in urine of animals. However, the values obtained for 2,5-DMP, although they were above the limit of detection for this method (LOD=17,7 nmol/mL), are beyond the limit of quantification (LOQ=52,6 nmol/mL) (Table 4.3).

Concerning 2,5-DMP levels, the animals of Group II present a lower urinary excretion of this pyrrole than those of Group I. This decrease (Table 4.3) was only quantified after the 3rd dose and follows the decrease of DMPN (Figure 4.10), being the levels of 2,5-DMP excretion much lower than those of DMPN.

Table 4.3 Urinary excretion of pyrrole compound 2,5-DMP of 2,5-HD exposed rats and co-exposed rats (2,5-HD+NAC). Data represents the mean ± SD (n=7).

Urinary excretion of 2,5-DMP (nmol/mL)		
Dose	**2,5-HD**	**2,5-HD+NAC**
1st	< LOQ (44.5 ± 7.4)	< LOQ (30.9 ± 1.1)
2nd	< LOQ (49.2 ± 7.2)	< LOQ (20.3 ± 6.4)
3rd	58.8 ± 5.6	< LOD
4th	61.1 ± 16.7	< LOD

4.4.2 LOCOMOTOR ACTIVITY ASSESSMENT

Two parameters (Fig. 4.13), ambulation (horizontal activity and number of crossings to different quadrants) and rearing (vertical activity and number of times where both forelegs were raised from the floor) were analyzed at two different time points: at pre-exposure (immediately before the first 2,5-HD dose) and after the last administration.

AMBULATION

REARING

Figure 4.13 Animals motor activity recorded during 5 min in an open field, by alteration on motor activity counts of two behavioral end points: rearing and ambulation.

In Figures 4.14 and 4.15 is represented the results of all groups. Dysfunction on ambulation and rearing in Group I are statistically significant (p<0.01) when compared with control group.

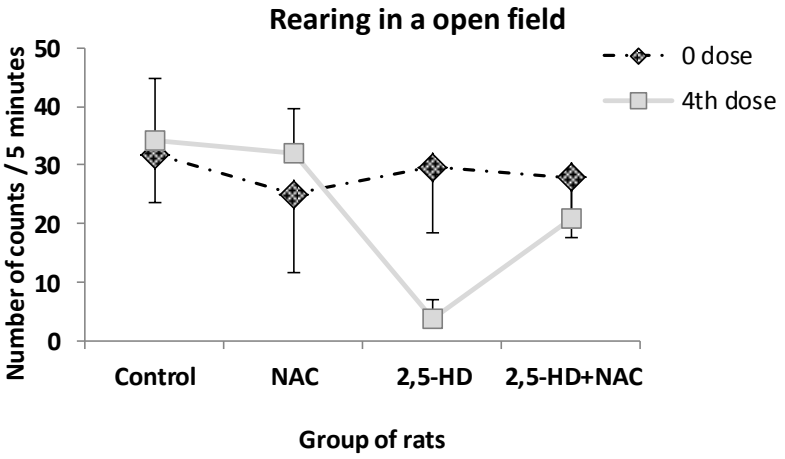


Figure 4.14 Support on hind legs in the open field (number of restraints on their hind legs for 5 minutes) of unexposed rats (control group), exposed to NAC, exposed to 2,5-HD and co-exposed to 2,5-HD+NAC. Data of 0 and 4 doses are expressed by the mean value + or - SD (n=7).

Additionally, in the group exposed to (2,5-HD+NAC) a significant recovery of motor activity was observed as compared to the group exposed to 2,5-HD alone, being the obtained values nearly similar to control values.

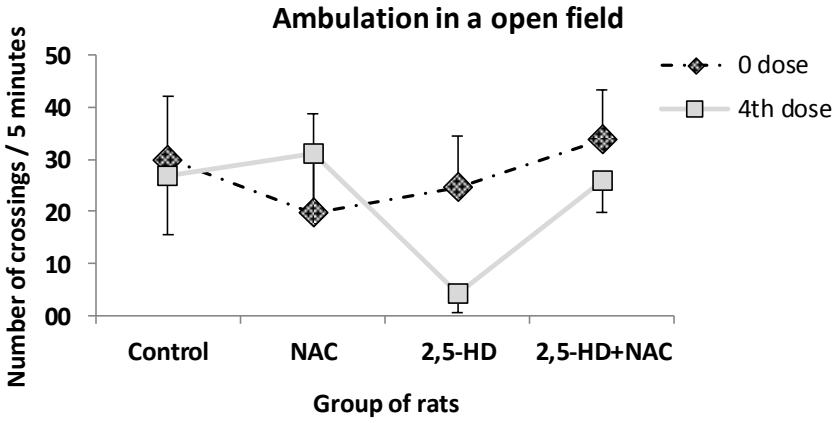


Figure 4.15 Passages in the open field (number of times that the markings are crossed for 5 minutes) of unexposed rats (control group), exposed to NAC, exposed to 2,5-HD and co-exposed to (2,5-HD+NAC). Data of 0 and 4 doses are expressed by the mean value + or - SD (n=7).

These figures illustrate a causal relationship between 2,5-HD exposure and the decrease of motor activity. The results of rearing and ambulation for the animal groups in study, for the fourth dose, are shown in Figure 4.16.

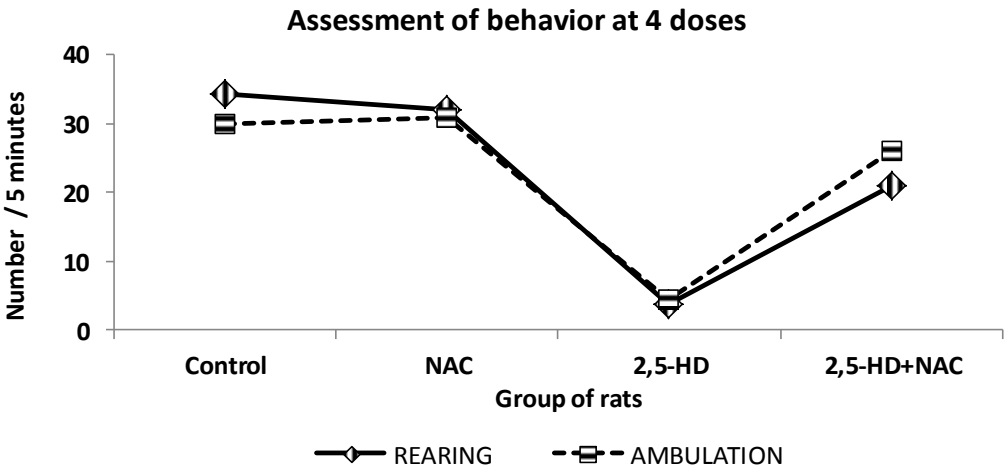


Figure 4.16 Results of rearing and ambulation for I (2,5-HD), II (2,5-HD+NAC), III (NAC) and IV (Control) Groups, after the fourth dose treatment.

4.4.3 CORRELATION BETWEEN URINARY BIOMARKERS LEVELS AND MOTOR ACTIVITY PERFORMANCE

To explore the toxicological meaning of the analyzed urinary biomarkers 2,5-HD, DMPN and DMPN NAC, we calculated the best correlation against the behavioral changes.

In Figure 4.17 it is observed a negative correlation between the urinary DMPN levels and motor activity, *i.e.*, the higher is the amount of urinary excretion of DMPN, the lower is the motor activity performance. On the other hand, a positive correlation was found between the motor activity functions and the urinary levels of DMPN NAC (Fig. 4.18).

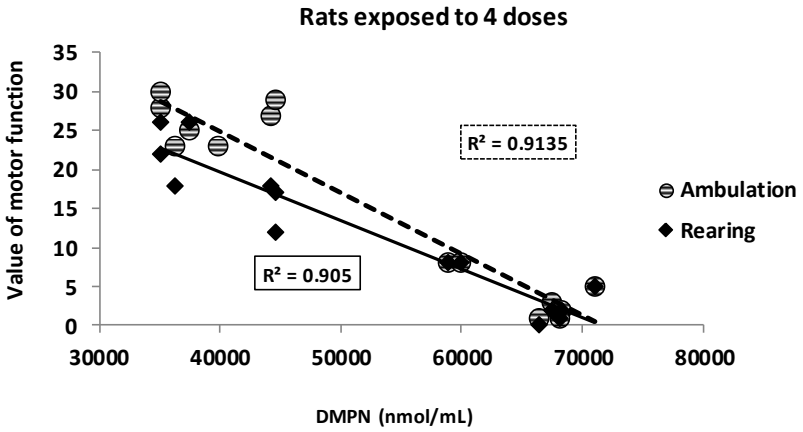


Figure 4.17 Correlation between ambulation and rearing, and pyrrole compound DMPN in the urine of rats treated with 4 doses of 2,5-HD and rats treated with (2,5-HD+NAC). For ambulation R^2=0.9135, p<0.01, for rearing R^2=0.905, p<0.01.

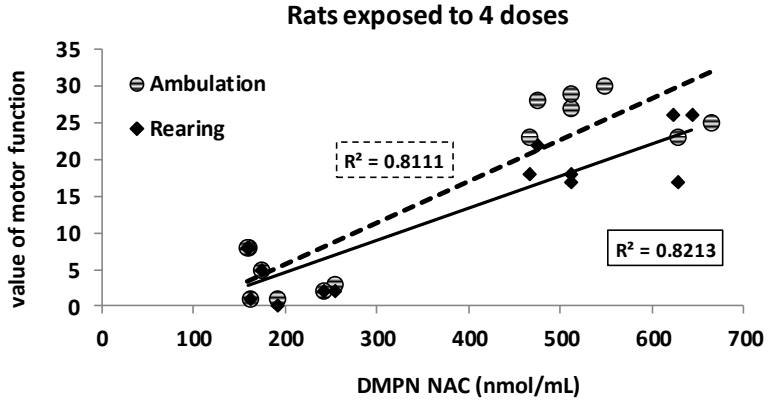


Figure 4.18 Correlation between the values of ambulation and rearing and DMPN NAC in the urine of rats exposed to 4 doses of 2,5-HD and group of rats co-exposed to (2,5-HD+NAC). For ambulation R^2=0.8111, p<0.05, for rearing R^2=0.8213, p<0.05.

In Figure 4.19 it is shown a negative correlation between urinary 2,5-HD levels and motor activities changes.

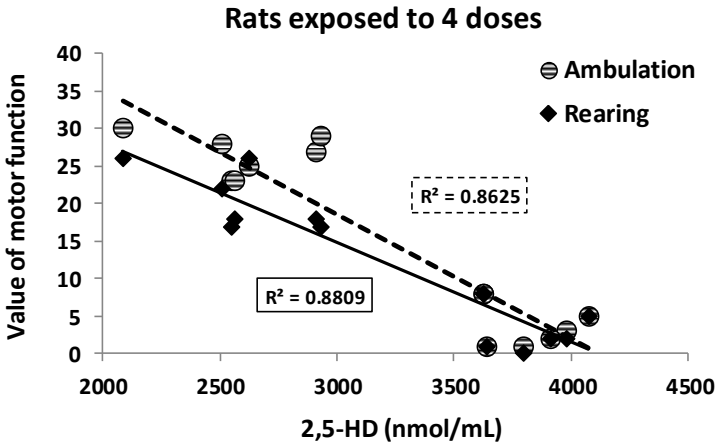


Figure 4.19 Correlation between ambulation and rearing with free 2,5-HD in the urine of rats, treated with 4 doses of 2,5-HD and treated with (2,5-HD+NAC). For ambulation R^2=0.8625, p<0.01, for rearing R^2=0.8809, p<0.01.

4.4.4 PHYSIOLOGICAL PARAMETERS

Ongoing assessment was performed during the experiment. Thus, to check the changes in rats body weight due to 2,5-HD exposure and the interference of NAC on these potential changes, we determined at the beginning and at the end of the experiment the body weight of all rats .

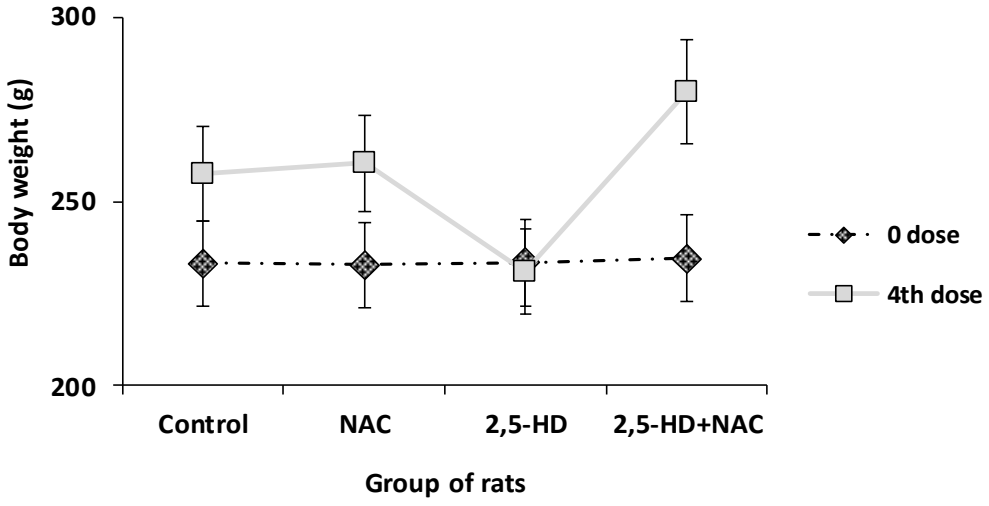


Figure 4.20 Body weight of the control group, rats exposed only to 2,5-HD, NAC and co-exposed (2,5-HD+NAC) to zero and fourth dose. Values are means ± SD (n=7).

Figure 4.20 shows no difference of body weight between controls Groups (III and IV). After the 4 doses, the group exposed to 2,5-HD evidences a body weight decrease comparing with all other groups. The co-exposed group to 2,5-HD+NAC evidences a tendency to a body weight increase.

4.5 DISCUSSION AND CONCLUSIONS

In this study the correlation between 2,5-HD, DMPN and DMPN NAC urinary levels and the motor activity performance in rats exposed to 2,5-HD and co-exposed to 2,5-HD+NAC were determined to clarify their use as biomarkers and to investigate the role of NAC in protecting against 2,5-HD neurotoxicity.

The interference of NAC on 2,5-HD neurotoxicity was evidenced by a significant decrease in urinary free 2,5-HD (Fig. 4.6) and DMPN levels (Fig. 4.10), a significant increase in urinary DMPN NAC (Fig. 4.12), and a significant recovery of neurobehavior motor activities in animals co-exposed (Fig. 4.16), when compared to their values in rats exposed to 2,5-HD alone.

The choice of behavioral endpoints to predict 2,5-HD neurotoxicity was based on their frequent use to identify chemical-induced alterations in nervous system functions, and to the knowledge that motor dysfunctions, as rearing and ambulation, could be used as sensitive indicators of 2,5-HD motor activity depression (Ladefoged et al., 1994; Kulig, 1996; Mateus et al., 2002). This preliminary finding indicates that for the 4 administrated doses of 2,5-HD, NAC protects against 2,5-HD neurotoxicity.

Figure 4.10 shows a significant increase of urinary DMPN in rats exposed to 2,5-HD as compared with control and a significant decrease of urinary excretion of DMPN in rats co-exposed to 2,5-HD+NAC, when compared to the groups exposed to 2,5-HD alone.
Based on the mechanism of 2,5-hexanedione neurotoxicity, it is established that 2,5-HD reacts with amino groups of lysine forming pyrrole adducts, thus, these results may be explained

considering that the formation of DMPN depends directly on the reaction between the γ-diketone and the ε-amino group of lysine in proteins.

In fact, our results show that in rats co-exposed to 2,5-HD+NAC (Fig. 4.6) it is observed a significant decrease of urinary free 2,5-HD as compared with its urinary levels in rats exposed to 2,5-HD alone. This finding is in accordance with the recovery of motor activity functions in the same co-exposed group (Fig. 4.16). It is well known that is the free 2,5-HD that reaches the target organs and is the responsible by its toxicity, thus, the decrease of free 2,5-HD levels probably explains the decrease of urinary DMPN levels and indicates the reduction of its neurotoxicity.

Our hypothesis is that, in (2,5-HD+NAC) co-exposed animals, free 2,5-HD may partly react with thiol (-SH) residues of Cys of NAC or sulfhydryl groups of GSH, before reaching the ε-amino groups of lysine residues of NFs.

Higher levels of urinary total 2,5-HD were found for the co-exposed group as compared with the group exposed to 2,5-HD (Fig. 4.7). This result may be explained considering that total 2,5-HD, corresponds mainly to the conjugated products of 2,5-HD biotransformation process suggesting that NAC may contribute to the 2,5-HD detoxification.

Concerning the quantification of urinary DMPN NAC, the co-treated rats, show a significant increase on its urinary excretion, as compared with its excretion in 2,5-HD treated rats (Fig. 4.12). Previous studies performed by Zhu and co-workers (1995), demonstrated that in the presence of biological thiols (GSH and cysteine) a mechanism of inhibition of auto-oxidative pyrrole-to-pyrrole cross-linking may occur, *via* thiol-to-pyrrole conjugation as well as through direct antioxidant action. Thus, the increase of DMPN NAC levels in urine of co-exposed animals may be associated with the inhibition of pyrroles auto-oxidation, remaining the DMPN free to adduct to NAC or GSH. In fact, since pyrrole formation itself is not sufficient to cause neuropathy, we suggest that NAC induces the increase of biological thiols which may contribute indirectly to prevent the progression of 2,5-HD induced neuropathy by promoting the detoxification of ROS, then reducing the probability of autoxidation of pyrroles, the subsequent pyrrolyl-pyrrole crosslinking and the development of neuropathies. This hypothesis may explain the recovery of motor activities in rats co-treated with 2,5-HD+NAC shown in Figs. 4.14 and 4.15.

On observing Table 4.3 we suggest that a marked decrease of urinary excretion of 2,5-DMP in rats co-exposed to 2,5-HD+NAC, may occur, although the values for the 1st and 2nd doses were below LOQ.

To understand the toxicological meaning of the urinary biomarkers excretion, in Figures 4.17, 4.18 and 4.19 we plotted the variation of the levels of the studied biomarkers and the motor activity functions in treated and co-treated rats. The best correlation was found for DMPN and motor activity (R^2=0.9135 for ambulation and R^2=0.905 for rearing), suggesting that this pyrrole compound may be proposed as a new sensitive predictive biomarker of 2,5-HD neurotoxicity.
Thus, the results suggest that at the studied conditions:

i) NAC protects against 2,5-HD neurotoxicity and

ii) DMPN may be selected as a new specific and sensitive biomarker of 2,5-HD neurotoxicity.

Nevertheless, the role of NAC in protecting against 2,5-HD neurotoxicity requires further investigation, thus, in Chapter 5 we performed a longer repeated co-treatment of rats with 2,5-HD+NAC.

CHAPTER 5

PROTECTION *vs* TOXICITY:
ROLE OF NAC
ON 2,5-HEXANEDIONE NEUROTOXICITY

5.1 INTRODUCTION

In vitro and *in vivo* studies reported the interference of NAC on several chemicals bioactivity underlying its protector effects against their toxicity.

Nevertheless, several authors refer different concentrations of NAC that induce toxicity using *in vitro* models. For example inhibitory effects on Na^+ absorption in human nasal epithelia were observed at a concentration near 10 mM NAC (Moschou et al., 2008), at concentrations greater than or equal to 30 mM, were reported toxic effects on diaphragm muscles (Khawli and Reid, 1994; Moschou et al., 2008) and at 5–10 mM concentrations, reductions in viability in rat and human aortic smooth muscle cells were documented (Tsai et al., 1996).

In vivo experiments were also reported using NAC. For example, 50 mg NAC/kg/day was orally, administrated in the prevention and treatment of acute lead intoxication in rats and the results suggest a decrease of lead absorption in the blood and tissues (Pande et al., 2001). More recently Lasram et al., (2014) studied the effects of NAC, *in vivo*, against pathological changes induced by malathion and Altinoz and Turkoz (2014) studied the protective role of NAC (250 mg/kg bw, via gavage) against acrylamide-induced genotoxicity and oxidative stress in rats.

Human studies were performed by Burgunder et al., (1989) to study the effect of oral administration of (2g) NAC in heathy volunteers after 2g of paracetamol administration and concluded that NAC induces glutathione synthesis and prevents the depletion of glutathione induced by paracetamol administration.

The objective of this work was to pursue the study of NAC interference on the biological activity of 2,5-HD using as tools the changes on selected biomarkers levels and the alterations on motor activity performance in rats treated with 2,5-HD and co-treated with 2,5-HD+NAC.

In Part I, we followed the same procedure of Chapter 4, extending the repeated co-treatment of rats with 2,5-HD+NAC and in the Part II we determined the levels of brain GSH and Cys in order to clarify the mechanism of NAC interference on 2,5-HD neurotoxicity.

PART I

URINARY BIOMARKERS ANALYSIS AND LOCOMOTOR ACTIVITY ASSESSMENT

5.2 MATERIAL AND METHODS

5.2.1 CHEMICALS

2,5-Hexanedione (2,5-HD, 99%) was purchased from Fluka, 2,5-dimethylpyrrole (2,5-DMP, 98%), Nα-acetyl-L-cysteine (NAC, 99%), Nα-acetyl-L-lysine (NAL, 98%) and γ-L-glutamyl-L-cysteinyl-glycine (GSH, 98%), were obtained from Sigma–Aldrich, 4-dimethylaminobenzaldheyde (DMAB, 99%) and boron trifluoride–methanol complex, ammonium acetate, acetonitrile *Lichrosolv* and ethanol *p.a*. were from Merck. Other reagents and solvents used, were of analytical grade and were purchased from reliable commercial sources.

5.2.2 INSTRUMENTATION

In this chapter was applied the same methodology reported in Chapter 4 to detect, identify and quantify the pyrrole compounds.

Thus, a Hitachi U-2000, UV–Vis spectrophotometer was used to confirm the presence of the pyrrole derivatives in rat urine after reaction with Ehrlich's reagent.

For the identification and quantification of pyrrole compounds, we used the ESI-LC-MS/MS instrumentation. The pyrroles analyzed were DMPN and DMPN NAC. In this Chapter, free 2,5-HD was determined by ESI-LC-MS.

LC–MS is compatible for aqueous matrices which enable to minimize or even eliminate sample preparation prior to analysis. The simplification of pre-analytical work reduces the time and the cost of analysis and minimizes the target analytes losses (Andreoli et al., 1998; Manini et al., 2004, 2006).

5.2.3 ANIMALS

Male Wistar rats (215 ± 20 g) from Charles River Laboratories® (Barcelona), were housed at controlled temperature, humidity and a 12-h light/dark cycle. As for the two previous studies, throughout the one week acclimatization period, and during all the *in vivo* assay, the general conditions of the animals were checked daily. Animals had free access to water and rat standard food (Letica Ref. IPM-R20). All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

5.2.4 RATS TREATMENT AND URINE COLLECTION

The same procedure was used as performed for the *in vivo* sub-acute study (Chapter 4). Before analysis, all urines were centrifuged and filtered on a 0.20 µm membrane (CHROMAFIL® Xtra), to remove particulate matter.

5.2.5 EXPERIMENTAL PROTOCOL

Table 5.1 shows the experimental design of animal administration.

Table 5.1 Animals assay protocol.

Group	Treatment solution[a]	Doses
HD (**I**)	400 mg 2,5-HD/kg bw/48-h	12 (HD)
(HD+NAC) (**II**)	400 mg 2,5-HD/kg bw/48-h + 200 mg NAC/kg/day	12 (HD) + 31 (NAC)
NAC (**III**)	200 mg NAC/kg/day	31 (NAC)
Control (**IV**)	Sterile saline/48-h	12 (saline)

(a) All treatment solutions were prepared freshly on a daily basis. 2,5-HD was administered *via* ip injection and NAC was administered *via* oral in drinking water (7 animals/group).

The concentrations of the solutions administrated were weekly adjusted according to the variation of body weight and calculated taking into account that the volume of 2,5-HD solution to administrate *via* ip, was 1 mL. However, even within each group, the differences in rats body weigh were taken in to account when were calculated the levels of urinary biomarkers.

5.2.6 DETERMINATION OF 2,5-HEXANEDIONE AND PYRROLE COMPOUNDS BY LC-MS/MS METHOD

2,5-Hexanedione

An individual standard solution of 2,5-HD (10 ppm), was infused into the mass spectrometer and scanned in the positive ion mode in order to achieve the best conditions of analysis (cone and capillary voltage) corresponding to higher signal/noise ratio to detect the molecular ion. The optimum conditions of analysis (cone voltage and the best collision energy) for 2,5-HD standard were selected, to obtain a fragmentation spectra characteristic of compound and to maximize the two main product ions signals (see Table 5.2). Mass of the precursor ion corresponds to the mass of the corresponding protonated molecule $[M+H]^+$. In Table 5.2 are presented the principal ion intensity values obtained in the MS/MS spectra for 2,5-HD commercial standard.

Table 5.2 Optimized conditions for studied compound, 2,5-HD. .

Rt (min)	**Precursor ion (*m/z*) $[M+H]^+$**	**Products ions, *m/z*** **(relative intensity)**	***Cone Voltage (V)***	***Collision Energy (eV)***	***MRM*1 transition**	***MRM*2 transition**	***MRM*1/*MRM*2 (8 < n < 10; RSD)**
12.4	115	**97**$_{(100)}$, 79$_{(27)}$, **69**$_{(46)}$, 55$_{(19)}$, 43$_{(76)}$	30	10	115>97	115>69	13.60 (3.8%)

Pyrrole Compounds

The quantification of pyrrole compounds was performed as reported previously, using *MRM*1 and *MRM*2 of each pyrrole compound (Chapters 3 and 4).

In brief, the mass spectrometer was operated in multiple reactions monitoring (*MRM*) mode. We use transition (*MRM*1) for quantification and (*MRM*2) for confirmation. The specificity of the method of analysis was guaranteed based on the comparison of: (1) the retention time of the of 2,5-HD standard solution and the target analyte in the urines (pyrrole compounds), and (2) *MRM*1/*MRM*2 ratios. For peaks of compound detected in samples, this ratio should not be outside the ± 20% range, considering results obtained with the standards analyzed in the same conditions. These criteria are according to the Commission Decision (2002/657/EC - *Concerning the Performance of Analytical Methods and the Interpretation of Results*).

5.2.6.1 Validation parameters

The calibration curves, for quantitative analysis, of 2,5-HD was calculated by the analysis of 8 standard solutions (concentration range of 0.5–100 mg/L). These were performed by spiking pooled control urines (urine of animals of Group IV) with 2,5-HD, in order to eliminate a possible matrix effect.

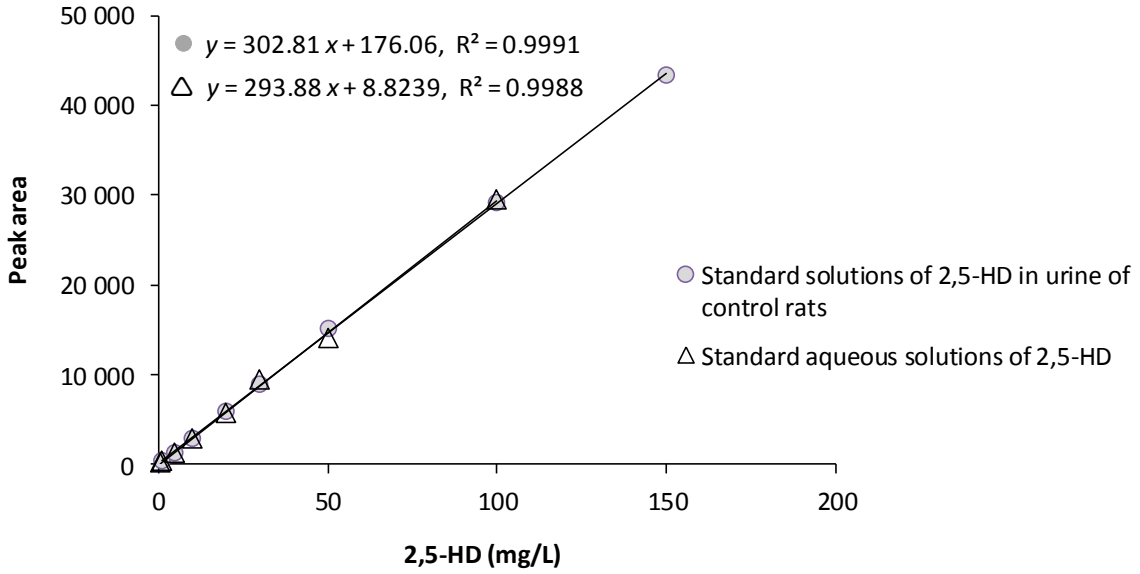


Figure 5.1 Calibration curves of 2,5-HD standard solutions in the concentrations range: 0.5-100 mg/L for aqueous solutions and 1-150 mg/L for standard solutions in urine.

Under the optimized conditions, the calibration curve of 2,5-HD was linear in the range of 0.5-100 mg/L. Squared Correlation Coefficients were 0.9988 and 0.9991 with water and with control urine, respectively (Fig. 5.1). The value obtained for matrix effect in urine solutions was 3%. Thus, for 2,5-HD, matrix effect during analyte ionization may cause suppression of the analyte signal.

The instrumental limit of detection for 2,5-HD, (LOD), was estimated considering the concentration of the compound that gives a signal that corresponds to three times the baseline noise (S/N≥3) and the instrumental limit of quantification (LOQ) as 3.3×LOD (S/N±10), as was calculated for 2,5-DMP (Chapter 3).

The limit of detection and quantification obtained for 2,5-HD, were LOD=14.6 nmol/mL and LOQ=43.8 nmol/mL. These values were estimated from S/N ratio of the respective MRM chromatograms, as derived from the analysis of standards. 2,5-HD quantification in urine of exposed and control rats was expressed in terms of nmol of 2,5-HD/mL.

The method presents a good repeatability at LOQ. Its value is below 10% (RSD=3.3%).

5.3 RESULTS

Analysis of urinary biomarkers and motor activity assays were performed in control, treated and co-treated animals.

5.3.1 IDENTIFICATION OF 2,5-HD IN RAT URINE

The ESI-LC-MS/MS analytical conditions described previously, allowed us to identify 2,5-HD in urine of rats treated and co-treated.

A chromatogram profile corresponding to 2,5-HD (retention time of 12.4 min) detected in MRM mode in urine of rat of Group I, is presented in Fig. 5.2.

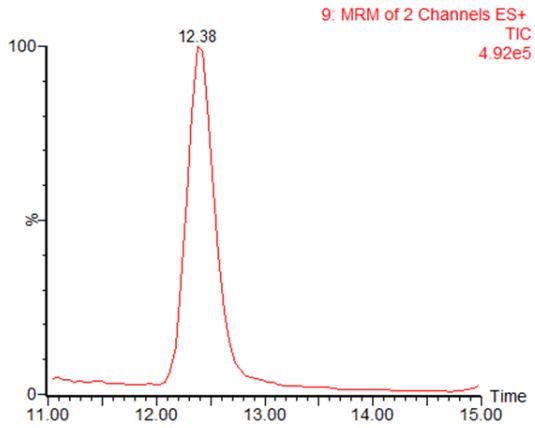


Figure 5.2 Chromatogram in MRM mode obtained after analysis of urine of a 2,5-HD exposed rat, for 2,5-HD compound (Rt=12.4 min).

The two most important transitions, existing in urine, for 2,5-HD: *MRM*1, 115>97 (quantification) and *MRM*2, 115>69 (confirmation), as well as the value of their intensities are shown in Fig. 5.3.

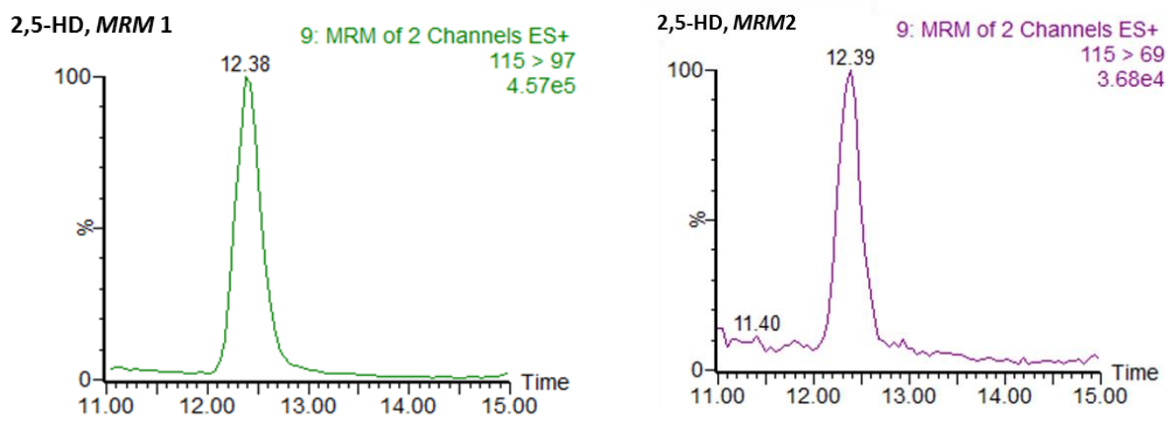


Figure 5.3 Chromatograms in MRM mode of the two most important transitions for 2,5-HD detected in urine of rat of Group I: *MRM*1, 115>97 (transition of quantification) and *MRM*2, 115>69 (transition of confirmation), as well as the value of their intensities, $4.57x10^5$ and $3.68x10^4$ respectively.

The product-ions mass spectrum from molecular ion of 2,5-HD, *m/z* 115, obtained through a urine of an exposed animal to 2,5-HD is presented in Fig. 5.4.

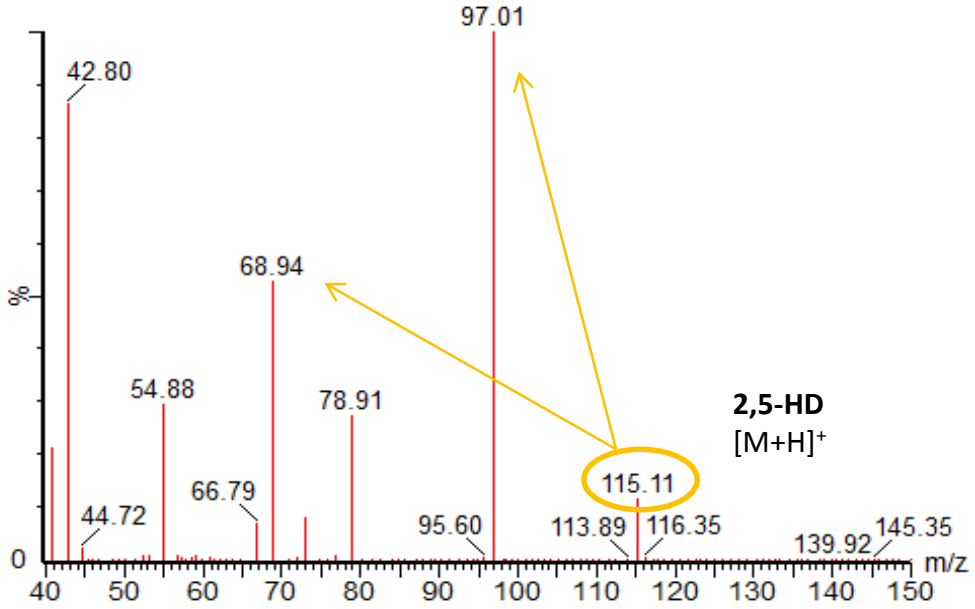


Figure 5.4 Product-ions mass spectrum from molecular ion of 2,5-HD, *m/z* 115, and the fragmentation ions *m/z* 43, 55, 69, 79 and 97. Spectrum obtained through an urine of an exposed animal to 2,5-HD.

5.3.2 QUANTIFICATION OF 2,5-HEXANEDIONE IN RAT URINE

The results obtained for free 2,5-HD are represented in Figure 5.5. It is shown a significant decrease (p<0.01) in urinary 2,5-HD levels in the co-treated rats (Group II) as compared with its levels in rats treated with 2,5-HD (Group I) after the 1st, 4th and 8th doses treatment. However, after the 12th dose, 2,5-HD levels for Group II are lower but no significant differences are found between the treated and co-treated rats.

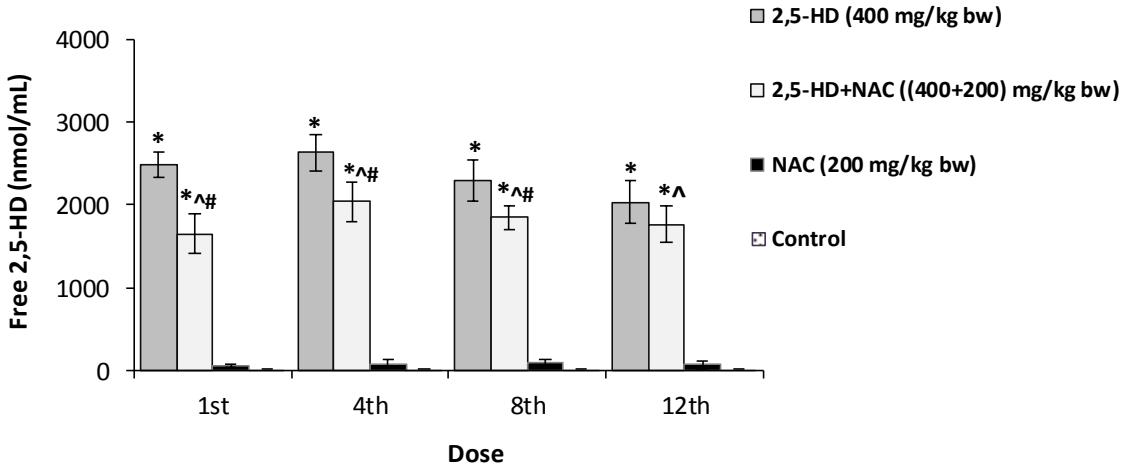


Figure 5.5 Urinary excretion of free 2,5-HD (expressed as nmol/mL) in rats treated with 2,5-HD (Group I), co-treated with 2,5-HD+NAC (Group II), treated with NAC (Group III) and saline treated (Group IV). Values represent means ± SD for n=7. All groups were compared by Mann–Whitney tests. (*) p<0.01 for Group I and II *vs.* Group IV; (^) p<0.01 for Group II *vs.* Group III and (#) p<0.01 for Group I *vs.* Group II.

5.3.3 QUANTIFICATION OF D PYRROLE COMPOUNDS IN RAT'S URINE

Detection by Colorimetric Method

The colorimetric test (Ehrlich's reagent) was also used to control the presence of pyrrole compounds in analyzed samples of urines of animals treated with 2,5 HD and co-treated 2,5-HD+NAC for repeated study.

This test allowed us to confirm that all animals exposed to 2,5-HD or co-exposed to 2,5-HD+NAC, presented in their urine pyrrole compounds.

5.3.3.1 Quantification of pyrrole compounds

The urinary levels of DMPN, along the 12 doses treatment is shown in Figure 5.6. In Group II the animals co-treated with 2,5-HD+NAC present a significant decrease (p<0.01) of DMPN, when compared with its levels in animals treated with 2,5-HD. However, this decrease is not significant after 12 doses of treatment. DMPN urinary values of control animals are not presented graphically as they are below the LOQ of the method.

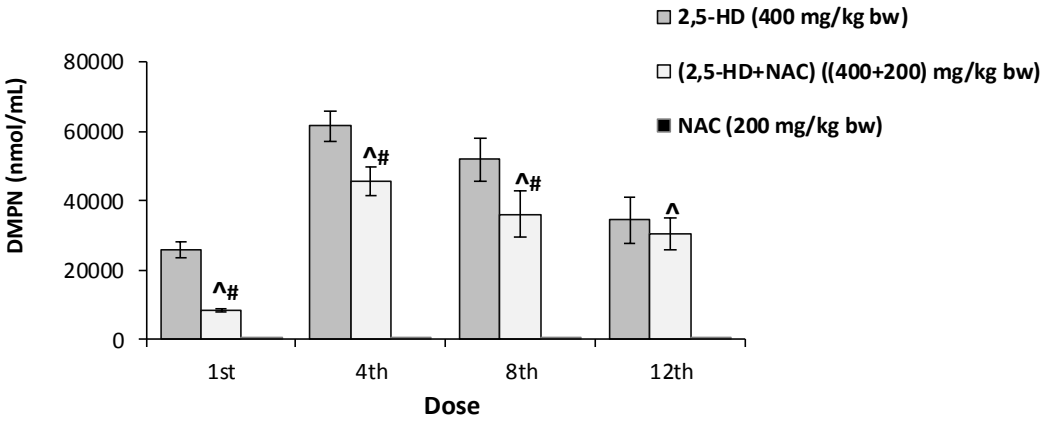


Figure 5.6 Urinary excretion of the pyrrole compound, DMPN, in 2,5-HD treated rats, (2,5-HD+NAC) co- treated rats and NAC treated rats. Data represents the mean ± SD for n=7. All groups were compared by Mann–Whitney tests. (^) p<0.01 for Group II *vs.* Group III, and (#) p<0.01 for Group I *vs.* Group II.

The urinary DMPN NAC levels along the study were also determined. In Figure 5.7 it is shown a significant increase (p<0.01) of urinary excretion of DMPN NAC in rats co-treated as compared with rats treated with 2,5-HD alone. The results for Group III (NAC treated) and Group IV (control) are below the detection limit of the method.

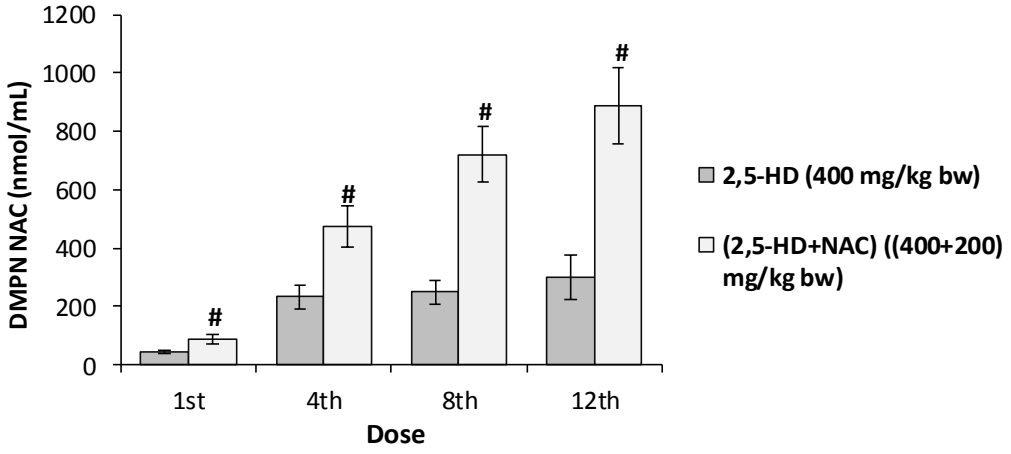


Figure 5.7 Urinary excretion of the pyrrole compound DMPN NAC in 2,5-HD treated rats and (2,5-HD+NAC) co-treated rats. Data represents the mean ± SD for n=7. The two groups of results were compared by Mann–Whitney tests. (#) $p<0.01$ for Group I *vs.* Group II.

5.3.4 LOCOMOTOR ACTIVITY ASSESSMENT

Motor activity performance in an open field, was evaluated through the assessment of two parameters: ambulation (horizontal activity) and rearing (vertical activity) which were obtained at different time points: at pre-exposure (before the first 2,5-HD dose) and after 4th, 8th and 12th administration.

After the 1th dose a significant ($p<0.01$) recovery of ambulation and rearing was found in Group II, (2,5-HD+NAC), as compared to the reduction of motor activities observed in Group I (2,5-HD). In addition, after the 8th dose a significant decrease ($p<0.01$) on motor activities in Group I and II when compared with Groups III (NAC) and IV (Control) was observed (Fig.5.8 and Fig.5.9).

In the same figures it is also shown after the 4th dose, co-treated animals (Group II), progressively approximate the behavioral performance of rats treated with 2,5-HD alone (Group I), being more evident after the 12 doses treatment.

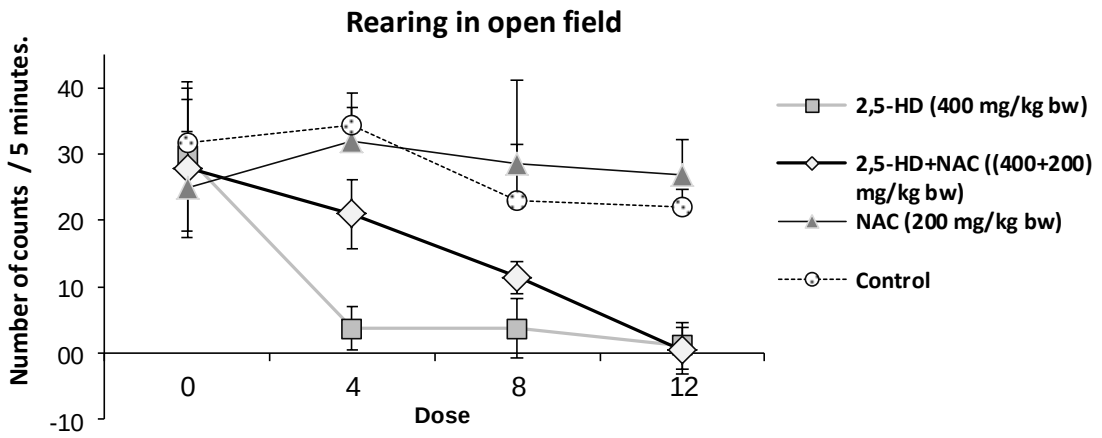


Figure 5.8 Support on hind legs in the open field (number of restraints on their hind legs for 5 minutes) of rats treated with 2,5-HD, co-treated with (HD+NAC), treated with NAC and untreated rats (control group). The results are expressed by the mean ± SD values, for n=7, and refer to the exposure of 0, 4, 8 and 12 doses. Groups (I+II) and (III+IV) of results were compared by Kruskall Wallis tests and are only statistically significant for 4th, 8th and 12th doses.

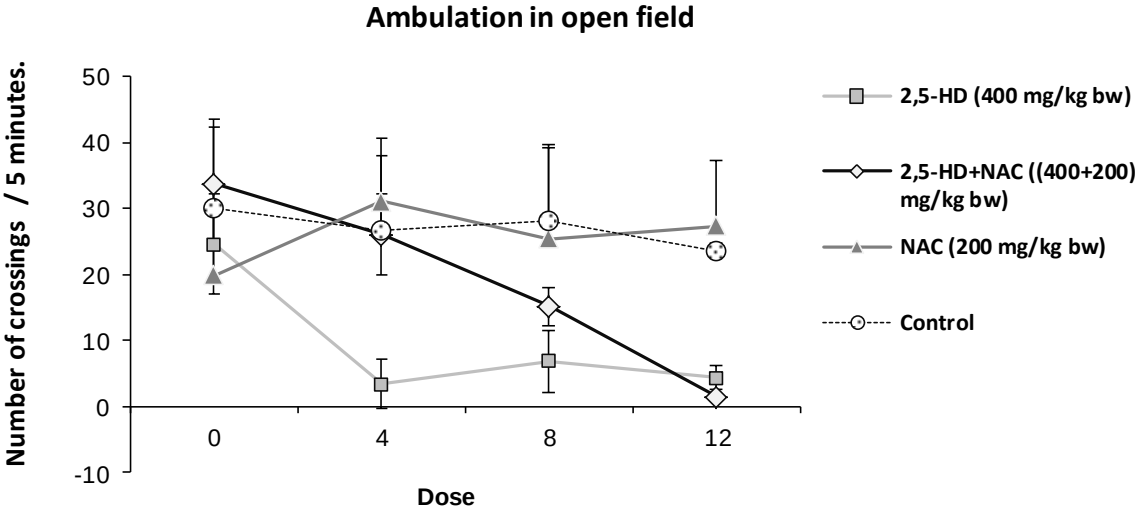


Figure 5.9 Passages in the open field (number of times that the square markings are crossed during 5 minutes) of rats treated with 2,5-HD, co-treated with (HD+NAC), treated with NAC and untreated rats (control group). The results are expressed by the mean ± SD values, for n=7, and are referred to the exposure of 0, 4, 8 and 12 doses. Groups (I+II) and (III+IV) of results were compared by Kruskall Wallis tests and are only statistically significant for 8th and 12th doses.

5.3.5 PHYSIOLOGICAL PARAMETERS

Ongoing assessment was performed along the study through a daily observation of animals in cages and through the determination on changes of rat's body weight in the 4 groups of rats.

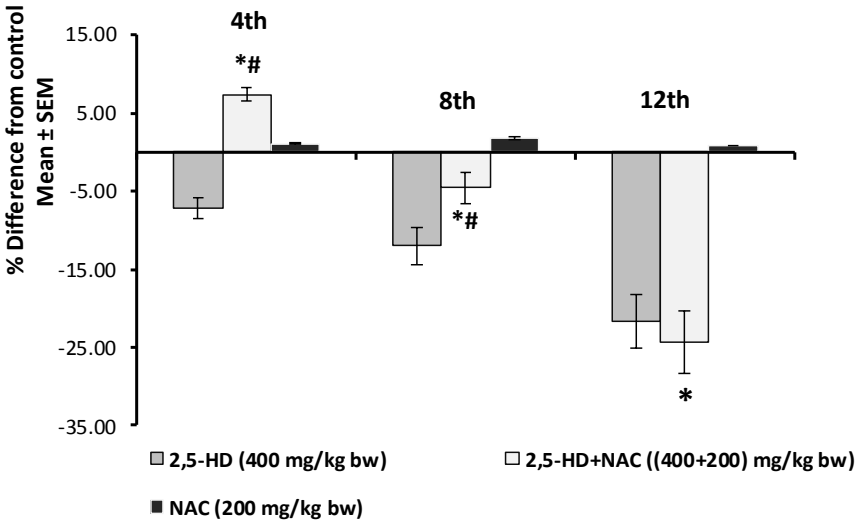


Figure 5.10 Difference of body weight from control group (%): rats treated only with 2,5-HD (Group I), co-treated with (2,5-HD+NAC) (Group II) and treated with NAC (Group III), for 4, 8 and 12 doses. Data represents the mean ± SEM for n=7. Results were compared by Mann-Whitney tests. (*) $p<0.01$ for Group II *vs.* Group III and (#) $p<0.01$ for Group I *vs*. Group II.

During the 4 weeks experiment, Group I evidences a tendency to a weight loss along the treatments. Group II evidences a tendency to a weight body increase over the 4th dose, a slight decrease on the 8th dose and a marked decrease in body weight at 12th doses.

5.4 DISCUSSON AND CONCLUSION

The role of NAC in protecting against 2,5-HD neurotoxicity was further investigated by increasing the number of administrated doses (12), in rats treated with 2,5-HD and co-treated with 2,5-HD+NAC.

To achieve this objective we applied the same methodology used in Chapter 4. Thus, determining the changes in free 2,5-HD, DMPN and DMPN NAC urinary levels and assessing the motor activity performance in rats treated with 2,5-HD (Group I), co-treated with 2,5-HD+NAC (Group II), treated with NAC (Group III) and saline treated (Group IV).

The interference of NAC on 2,5-HD neurotoxicity was evidenced by a significant decrease in urinary free 2,5-HD and DMPN levels until 8th dose in Group II as compared with Group I. However, this decrease was no longer significant after 12 doses treatment. A significant increase of urinary DMPN NAC in Group II as compared with Group I for all the administrated doses it was also observed.

In this experiment, the behavioral data reported in Chapter 4 was confirmed because it was also observed a significant recovery on motor activity performance in Group II until the 4^{th} dose administration. However, it is evident that this protector effect is decreasing progressively over the time of co-treatment and from the 8^{th} dose there isn't any significant difference in behavioral performance between the Group I and Group II. Moreover, at the 12^{th} dose it appears that there is a reversal of the NAC effect likely producing an increase of toxic effect.

Concerning the observed significant decrease ($p<0.01$) of urinary free 2,5-HD and DMPN levels (Figs. 5.5 and 5.6), in the previous chapter we justified the protective role of NAC against the neurotoxicity of 2,5-HD until the 4 doses administration. In brief the decrease of urinary DMPN levels in rats treated with the mixture may be a consequence of the observed reduction of free 2,5-HD that may be partly due to its higher probability to react with cysteine thiol (SH) residues of NAC or sulfhydryl groups of GSH, before reaching the amino groups of lysine in NFs. In fact, the formation of DMPN depends directly on the reaction between the γ-diketone and the ε-amino group of lysine in proteins. In particular, the chemical adduction of proteins that are critical to the neuronal functions may be dictated by the position of the aforementioned amino residues

within (neuro)-protein sequences and their active/catalytic sites (Tshala-Katumbay et al., 2009a). The interactions of the hard electrophile 2,5-HD, with the hard nucleophile, ε-amino groups on lysine residues of axon cytoskeletal proteins is based on HSAB theory, which may also explain the interference of NAC on 2,5-HD neurotoxicity. In fact, the decrease in urinary concentration of DMPN in co-treated animals must also take into account the low nucleophilicity of ε-amino group of lysine (+1) at physiological pH, which means that the formation of adducts with 2,5-HD is very slow. Meanwhile, the reaction between 2,5-HD or pyrrole compound and cysteine sulfhydryl protonated (0) thiol may occur, as this thiol is present at higher levels in co-treated animals.

Figures 5.8 and 5.9 evidence that until the 4th dose of co-treatment, NAC presents a protector effect against 2,5-HD neurotoxicity. However after the 4th dose, there is an attenuation of protector effect and possibly emerges a toxic effect which is shown after 12 doses co-treatment.

This toxicity is evidenced by motor activity assessment as well as by the decrease of body weight of co-treated animals. Ongoing assessment was performed to determine the changes on rats body weight along the 4 weeks study. In Fig. 5.10 it is shown that at 4 doses there is an increase on the body weight of animals of Group II, as compared with Group I, where it is observed a marked decrease of rat body weight as compared with Groups III and IV.
At 12 doses, the Groups I and II have a significant decrease (p<0.01) of their body weights, which is an alert to the potential toxicity induced by 2,5-HD as well as by the 2,5+NAC treatment.

In Figs. 5.8 and 5.9 it is also shown that at 12 doses the two groups (I and II) show significant motor activity dysfunctions. It is well known that 2,5-HD induces neurotoxicity which confirm our results in Group I. The same is not true for the Group II where no data is reported concerning the interaction between 2,5-HD and NAC at any dose. Our results show that after the 4th dose, there is a progressive reduction of protection effect and a slight increase of neurotoxicity in rats co-treated with 2,5-HD+NAC (Group II). In fact, through the behavioral tests (ambulation and rearing), we also verified that the animals exposed to 12 doses of 2,5-HD exhibited hind limb muscle weakness due to gait abnormality and decrease in grip strength and the extensor thrust response. Moreover, co-treated animals, for the same time of exposure, exhibited gait

abnormalities (ataxia, splayed hind limbs), in conjunction with increased landing hind foot spread and decreased hind limb grip strength and extensor thrust, *i.e.*, hindlimb landing foot spread was not affected by 2,5-HD treatment. On the other hand, the group treated with NAC alone (Group III) did not present any toxicity which may suggest that the toxicity of Group II is induced by the formation and/or accumulation of a new neurotoxic compound.

Taking this into account, we also determined the changes on urinary excretion of DMPN NAC and it was found that in Group II (co-treated with NAC), there is a significant increase ($p<0.01$) on its levels as compared with the excretion in Group I (treated with 2,5-HD alone). Additionally, in Group II, there is a dose dependent increase on its excretion along the 12 doses treatment (Fig. 5.7). According with Zhu et al., (1995, 1997) DMPN NAC, a non-cross-linked product, is a more stable compound than other compounds resulting from 2,5-HD action treated with amino acids, peptides and proteins, and more recently it was referred (LoPachin and Gavin, 2012) that due to its great stability, adducts formed between electrophile DMPN and sulfhydryl thiolate may be irreversible. In this context, knowing that DMPN NAC, is a more stable compound than DMPN, we may wonder if it may accumulate in nervous system, inducing delayed toxicity in animals co-treated with 2,5-HD+NAC.

This suggestion is supported by LoPachin and Gavin (2012), that investigated acrylamide adducts with thiolate group, and concluded that CNS nerve terminal dysfunction occurs at a cumulative adduct level of 350–500 pg Cys adduct/μg protein. As intoxication continues and adduct formation exceeds this threshold, the pool of dysfunctional proteins increases proportionately and the related presynaptic processes are progressively disabled, leading to the characteristic cumulative neurotoxicity of acrylamide (LoPachin et al., 2006).

However, neurotransmission is a complex process that is highly regulated by NO signaling being NO a biological electrophile that forms reversible adducts with sulfhydryl thiolate groups (S-nitrosylation) on proteins. NO signaling transiently decreases synaptic strength by reversibly inhibiting the function of several proteins involved in the synaptic vesicle cycle (Kiss, 2000; Kiss and Vizi, 2001; LoPachin and Barber, 2006; Vincent, 2010; LoPachin and Gavin 2012). Consequently, other hypothesis is that the electrophilic neurotoxicant DMPN, selectively adducts NO-receptor thiolates in catalytic triads which results in loss of neurotransmission, having substantial implications for protein function and subsequent presynaptic toxicity.

On the other hand, a large number of structurally diverse chemicals are electrophiles and cause synaptic dysfunction by unknown mechanisms. Highly nucleophilic Cys thiolate groups within synaptic proteins represent targets for electrophilic neurotoxicants that share the ability to adduct or otherwise modify nucleophilic sulfhydryl groups. Thus, electrophilic neurotoxicants may produce synaptic toxicity by modifying these thiols since most proteins contain Cys residues. These sulfhydryl thiolate sites regulate protein activity by playing a direct role in the enzymatic catalytic process and a broad spectrum of synaptic activities by acting as acceptors for nitric oxide (NO), and other redox modulators (H_2O_2) that transiently regulate enzyme function (LoPachin et al., 2008b, 2009a,b; Jones, 2010; LoPachin and Gavin, 2014).

In conclusion, we hypothesize that the co-treatment of rats with 2,5-HD+NAC have a dual opposite dose dependent effect: firstly inhibiting the formation of DMPN crosslinks in axonium neurofilaments and protecting against 2,5-HD neurotoxicity, secondly accumulating the stable DMPN NAC adducts which after reaching a threshold value may induce neurotoxicity by unknown mechanisms.

PART II

DETERMINATION OF THIOLS IN URINE AND IN BRAIN TISSUE

5.5 INTRODUCTION

The brain has a large potential oxidative capacity, however a limited ability to counteract oxidative stress (Calabrese et al., 2003; Poon et al., 2004a; Halliwell, 2007). Within the cell, reactive oxygen species (ROS) are physiologically present at minimal concentrations, in normal conditions, and there is a steady-state balance between pro-oxidants and antioxidants which is necessary to ensure optimal efficiency of antioxidant defenses (Calabrese et al., 2004a,b; Forman et al., 2004; Poon et al., 2004b). However, when the rate of free radicals generation exceeds the capacity of antioxidant defenses, oxidative stress ensues with consequential severe damage to proteins and lipids, and has been implicated in mechanisms leading to neuronal cell injury in various pathological states of the brain, including neurodegenerative disorders (Calabrese et al., 2008).

The neurotoxicity of 2,5-HD was previously described (1.2.1 of Chapter 1) and the formation of pyrroles which readily autoxidize may bind to other lysine amino groups inducing crosslinks in neurofilaments, the responsible by 2,5-HD neuropathogenic toxicity (Genter et al., 1988; Amarnath et al., 1994; LoPachin and Lehning, 1997). In this context, the inhibition of pyrroles autoxidation may be promoted by thiol compounds, knowing the high affinity of pyrroles for the sulfhydryl groups in proteins, peptides or aminoacids. NAC, a thiolic antioxidant, acts as a precursor of the natural antioxidant glutathione that acts as hydrogen donor for free radicals since the S-H bond is relatively weak, and may also protect against oxidant damage (Zhu 1995; Banaclocha et al. 1997; Ayoama et al., 2008).

The main objective of this part of the work was to evaluate the effect of NAC in the levels of reduced glutathione (GSH) and Cys in brain of rats treated (Group I) and co-treated (Group II) in order to clarify the mechanism of interaction of NAC with 2,5-HD.

5.6 MATERIALS AND METHODS

5.6.1 ANIMALS

Experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals as stated in the beginning of Part I.
The experimental protocol of animals study was presented previously in Table 5.1.

5.6.1.1 Tissue collection

Animals were anesthetized 24 h after the last dose with diethyl ether. Immediately after, brain was removed, weighed and stored at −80°C, until analysis.

5.6.2 CHEMICALS

2,5-Hexanedione (2,5-HD, 99%) was purchased from Fluka, 2,5-Dimethylpyrrole (2,5-DMP, 98%), Nα-acetyl-L-cysteine (NAC, 99%), L-cysteine (L-Cys, ≥97%), Nα-acetyl-L-lysine (NAL, 98%), Cysteamine (98%), Tri-n-butylphosphine (TBP, 97%), N,N-dimethylformamide (MDF, ≥99,9%), 7-Fluorobenzofurazan-4-sulfonic acid ammonium (SBD-F, 98%) and Glutathione, in reduced form **(**GSH, 98%), were obtained from Sigma-Aldrich. Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

5.6.3 INSTRUMENTATION

To analyze thiol compounds in brain tissue, liquid chromatography (HPLC) with fluorescence detection was performed.

The HPLC system (Agilent 1100 Series) consisted of a G1311A Pump, a G1328B Injector, a spectrofluorophotometer (FLD) G1321A operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm and a UV-Vis Detector G1314A. The LC separation was performed on a reversed-phase column (LiChrospher® RP18, 125 × 4; 5.0 μm; Merck®) using an injection volume of 20 μL and a 0.1 N (pH 2.1) potassium dihydrogen phosphate buffer solution, as mobile phase at a flow rate of 1.0 mL/min, on isocratic elution.

Acquisition and quantification of the peaks from the HPLC system was performed by data acquisition programme software ChemStation®.

5.6.4 DETERMINATION OF THIOLS

The quantification of thiols in brain samples was performed using the internal standard method (0.58 mM cysteamine).

Derivatization, prior to chromatographic analysis, was performed to render thiols specifically detectable for fluorescence, which is a more sensitive tool for compounds detection. Thiols reacted with SBD-F (7-Fluorobenzo-2-Oxa-1,3-Diazole-4-Sulfonic Acid) in controlled conditions, allowing to transform them into fluorescent compounds.

Low levels of GSSG need to be accurately determined, thus, oxidation of GSH to GSSG has to be prevented during sample preparation and derivatization. This is particularly important since an alteration in the GSH/GSSG ratio is an indicator of oxidative stress.

5.6.4.1 Brain Tissues

Tissues samples were homogenized and weighed in individual samples of about 50 mg for an eppendorf. One mM of borate buffer, 2 mM EDTA (pH 9.5) in a volume 10 times greater than the weight of the sample brain, was added. Thereafter, each sample was subjected to sonication (30 cycles; Condition: Cycle 0.5, range 100). Withdrew 100 µL of each sample and proceeded to the technical derivation.

5.6.4.2 Derivatization reaction

To 100 µL of sample or standard were added 10 µL of internal standard (Cysteamine) and 10 µL of TBP in DMF (extemporaneous solution). It was placed at 4°C for 30 min. To the mixture, a 100 µL of 10% TCA (1 mM EDTA) was added and then was centrifuged at 25 000 *x g* for 10 min. A 50 µl was removed from the supernatant.
To ensure an alkaline pH, necessary to facilitate the derivatization reaction, a 10 µL of NaOH, 1.55 M was added. After that, 100 µL of borate buffer, 125 mM (4 mM EDTA, pH 9.5) and 50 µl of SBD-F (extemporaneous solution) were added to supernatant. The mixture was heated at 60°C. After one hour, the samples showed a yellowish color and were placed on ice until injection.

After sample preparation, we proceeded to its analysis by HPLC to determine thiols brain content.

5.6.4.3 Validation parameters for HPLC Method

The calibration curves for quantitative analysis, of thiol compounds in brain tissue, were obtained by the analysis of standards solutions, (n=7), of GSH and Cys in the concentration range between 0.05 mM and 0.5 mM. These standard solutions were prepared from stock solutions diluted with 1 mM borate buffer (2mM EDTA, pH 9.5).
For reduced GSH the coefficients, R^2, ranged between 0.9982 and 0.9977 and for L-Cys between 0.9979 and 0.9964. Under the optimized conditions for quantitative analysis of thiol compounds, calibration curves were linear in the ranges of concentrations utilized.

Instrumental limit of detection (LOD) and quantification (LOQ) of the assay for Cys and GSH was estimated from the standard deviation of the intercept response and the Slope, of calibration curves, exactly in the same way that was explained in 4.3.6.3 for 2,5-HD (Chapter 4). Results are presented in Table 5.3.

Table 5.3 LOD and LOQ values for GSH and Cys for HPLC method.(σ and S were obtained by SPSS SATISTICS® version 21). .

Compound	Values	LOD (µM)	LOQ
GSH	S=7.636771 σ=0.061406	**26.53**	**79.60**
Cys	S=1.897216 σ=0.016616	**28.90**	**86.70**

In every analytical run were included some calibration standards. All of the derivatized samples were kept in sample vials and reinjected periodically onto the HPLC system until significant changes in peak areas were noticed.

5.7 RESULTS

The chromatogram obtained with standard solutions of GSH and L-Cys (0.1 mM) and internal standard (IS) cysteamine (0.58 mM) is shown in Figure 5.11. It is possible to see peaks corresponding to L-Cys (Rt=3.030 min), and GSH (Rt=15.449 min) standards and for the cysteamine (Rt=4.064 min).

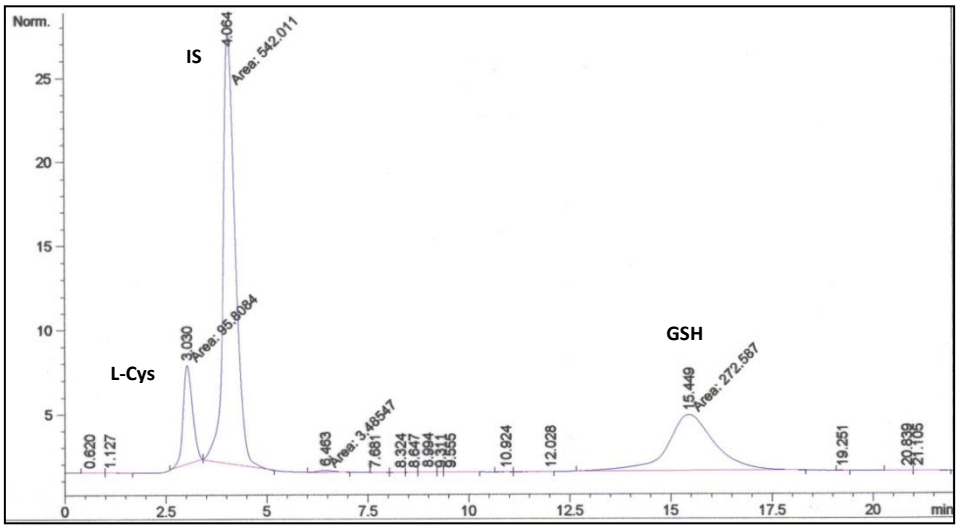


Figure 5.11 Chromatogram obtained with standard solutions of GSH, L-Cys (0.1 mM), and cysteamine (IS).

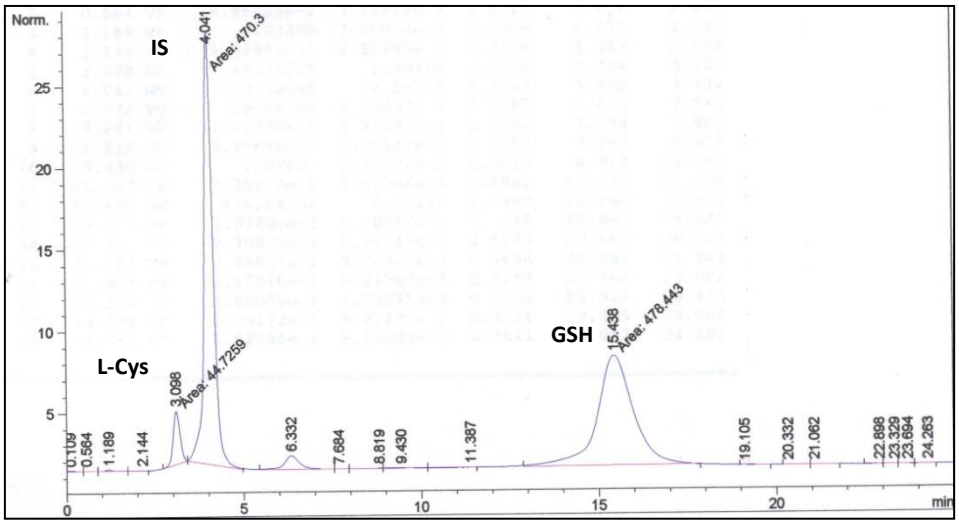


Figure 5.12 Chromatogram obtained for a sample of brain tissue analyzed, referring to the group co-exposed to 2,5-HD+NAC (400+200) mg/kg bw.

In Figure 5.12 is shown the chromatogram obtained with a sample of brain tissue analyzed, referred to the Group II.

5.7.1 DETERMINATION OF THIOLS

In Figure 5.13 Group IV (control) presents the lowest values of Total GSH and Reduced GSH. However, this group doesn't present the lowest values for Cys. The lowest levels of Cys are seen in Groups II and III (co-treated with NAC and treated with NAC).

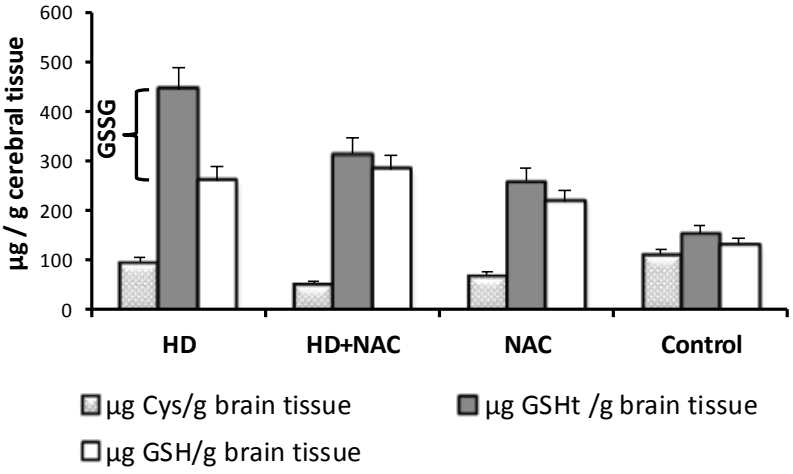


Figure 5.13 Concentration of Total GSH (GSHt), Reduced GSH (GSH) and Cys, determined in tissues from the brains of rats treated with 2,5-HD (Group I), co-treated with 2,5-HD+NAC (Group II), treated with NAC (Group III) and control (Group IV). (Columns are mean ± SD, n=7).

In the same figure it is shown that Group I presents the highest value of total GSH (GSHt) due to the highest value of GSSG as compared with the other groups

The concentration of Total GSH, Reduced GSH (GSHred) and Oxidized GSH (GSSG), determined in tissues from the brains of rats treated with 2,5-HD (Group I), co-treated with 2,5-HD+NAC (Group II), with NAC (Group III) and Control (Group IV) are shown in Fig. 5.14.
Group I presents the highest values of Total GSH and GSSG. The lowest levels are seen in Group IV.

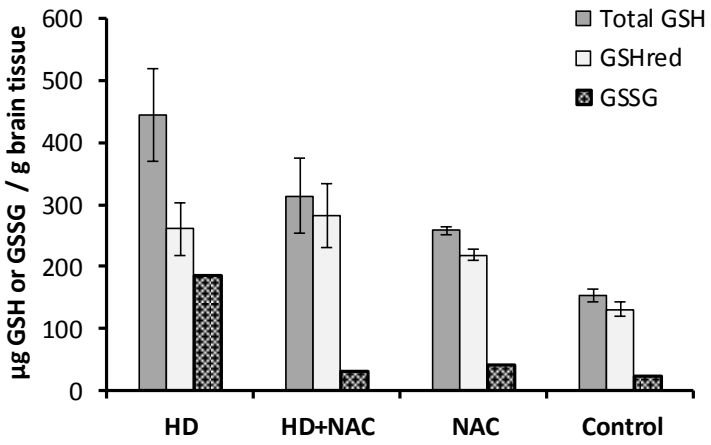


Figure 5.14 Concentration of Total GSH (GSHt), Reduced GSH (GSHred) and Oxidized GSH (GSSG), determined in tissues from the brains of rats treated with 2,5-HD (Group I), co-treated with 2,5-HD+NAC (Group II), with NAC (Group III) and Control (Group IV). (Columns are mean ± SD, n=7).

5.8 DISCUSSION AND CONCLUSION

In this work, we investigated the mechanism of interaction of NAC with 2,5-HD through the determination of brain GSH and Cys levels in rats treated with 12 doses of 2,5-HD+NAC.

NAC has antioxidant potential and its main metabolite, Cys, a precursor in the biosynthesis of reduced glutathione, is an extremely important intracellular and extracellular antioxidant (Bridgeman et al., 1991). Several authors, referred that Glutathione found in mammalians cells exists mainly in the reduced state (GSH), with <5-10% of the total existing as glutathione disulfide (GSSG) under normal physiologic conditions.

In Figures 5.13 and 5.14 it is shown that, Group II, Group III and Group IV present brain glutathione disulfide (GSSG) levels near 10, 15.4 and 15.0% of Total GSH respectively, being the highest brain GSSG concentrations found in Group I, approximately 42% of Total GSH. In addition, in Fig. 5.13 both groups exposed to NAC (II and III) have a lower value for Cys which

is probably due to its conversion to cystine. In fact, elevated tissue Cys levels should be avoided because they may lead to autoxidation of Cys to form cystine and ROS (Aoyama et al., 2008).

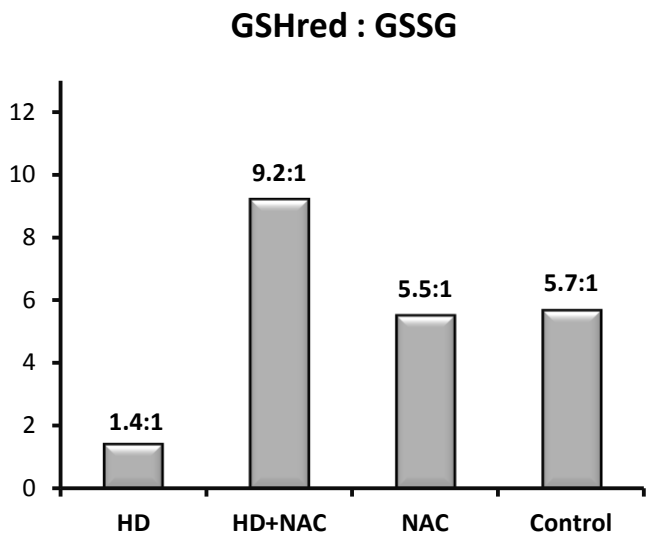


Figure 5.15 Ratio between GSH in reduced form and GSSG, in brain tissues of Groups I (2,5-HD) Group II (2,5-HD+NAC), Group III (NAC) and Group IV (Control).

In Figure 5.15, the lowest ratio of GSH/GSSG is observed in Group I, whereas in Group II the highest ratio of GSH/GSSG was found, suggesting that NAC induces GSH recovery.

In conclusion, our results didn't clarify how NAC interferes on 2,5-HD neurotoxicity as the increase of brain GSH/GSSG ratio in rats co-treated with 12 doses of 2,5-HD+NAC(Group II), only confirms its brain antioxidant function. However, NAC doesn't induce the recovery of neurobehavioral dysfunctions in the same rats (Group II), which means that its antioxidant properties are not correlated with its protector effects against 2,5-HD neurotoxicity after 12 doses of co-treatment. Moreover, the levels of brain Cys are not a good indicator to evaluate the role of NAC in 2,5-HD toxicity .

Other *in vivo* and *in vitro* experiments are programmed to clarify the mechanism of NAC interaction with 2,5-HD.

CHAPTER 6

PRELIMINARY STUDY TO IDENTIFY URINARY PYRROLE COMPOUNDS IN HEALTHY INDIVIDUALS AND IN PATIENTS WITH NEURODEGENERATIVE DISEASES

6.1 INTRODUCTION

In this thesis we proposed to select biomarkers to control cumulative n-hexane exposure and predict its induced neurotoxic effects.
In the previous chapters, we identified and quantified several urinary pyrrole compounds in rats treated with 2,5-HD and co-treated with 2,5-HD+NAC. By analyzing the results obtained it was possible to select DMPN and DMPN NAC as specific biomarkers of 2,5-HD exposure.

In this Chapter 6 we propose to investigate if the selected biomarkers could be identified in

A - An healthy population

B - Patients with neurodegenerative diseases

Several studies support this experimental proposal:

A - Healthy population

The endogenous production of n-hexane, may explain the appearance of low levels of 2,5-hexanedione in the urine of subjects unexposed to this solvent (Fedtke and Bolt, 1986a). Bavazanno et al., (1998) also refer the presence of 2,5-HD in subjects who have not been occupationally exposed to the above-mentioned solvent. For this purpose they postulated both an exogenous origin related to n-hexane micropollution and an endogenous source based on the hypothesis that 2,5-HD can act as an intermediate catabolite in some biochemical physiological processes. Perbellini et al., (1993), report results regarding physiological aspects of 2,5-HD excretion in subjects not occupationally exposed to n-hexane, proposing that it is a product of intermediate metabolism in the human body and only a minimal part could derive from n-hexane as a ubiquitous micropollutant. Finally, Persson et al., (2013), reviewed the urinary 2,5-HD levels in the general population and concluded that is detected in low concentrations (Table 6.1).

Table 6.1 Urinary levels of 2,5-hexanedione (2,5-HD) in general population.

Country	(men:women)	2,5-HD mean ± SD mg/L	2,5-HD range mg/L	Method	Reference
Germany	8:4	0.45 ± 0.20	0.12-0.78	GC-MS	Fedtke and Bolt, 1986a
Italy	10	0.49 ± 0.14	0.32-0.64	GC-FID	Perbellini et al., 1986
Japan	55:0	1.47 ± 0.60	-	GC-FID	Kawai et al., 1990
Japan	53:0	0.33 ± 0.47	-	GC-FID	Kawai et al., 1991
Italy	20:20	0.47 ± 0.21	0.10-1.00	GC-FID	Bavazzano et al., 1998

Kessler et al., (1990), reported that endogenous and exogenous 2,5-HD originates pyrrole substances that were identified as PLS "pyrrole-like substances" and the total pyrrole values in urine of rat and man exposed and not exposed to n-hexane were detected by Ehrlich's reagent.

B - Patients with neurodegenerative diseases

The etiology of most neurodegenerative disorders is multifactorial and it is general consensus that a convergence of factors (*e.g.*, genetic, environmental and age related), possibly specific to each disease, culminate in a common pathophysiological cascade involving oxidative stress and lipid peroxidation (LoPachin et al., 2008b). Damage to tissue biomolecules, including lipids, proteins and DNA, by free radicals is postulated to contribute importantly to the pathophysiology of oxidative stress (Migliore and Coppedè, 2009; Persson et al., 2013).

Concerning lipid peroxidation it is primarily initiated with hydrogen capture of a fatty acid by radical oxygen, followed by the formation of hydroperoxides. The degradation of these hydroperoxides results in a chain reaction producing various compounds including carbonyl groups mainly aldehydes and ketones (Yin and Chen, 2005). One of the carbonyl compounds which may be formed during lipid peroxidation is γ-diketone, 2,5-HD (Fedtke and Bolt 1986a).

In this context, we propose the identification of urinary γ-diketone derivative pyrroles in health individuals and in patients with neurodegenerative diseases, to investigate their use as one of the biomarkers of neurodegenerative diseases.

6.2 MATERIAL AND METHODS

The study was approved by the Research Ethics Committee of Casa de Saúde das Irmãs Hospitaleiras da Idanha, where the subjects were inpatients, and the patients' family members signed an informed consent form for their participation (Annex I of this chapter).

The study included 11 patients: 2 men and 9 women. The average age was 78.8±7.6 years.
In the group of patients 5 had Alzheimer Disease (AD), 4 had Vascular Dementia (VD), 1 had Parkinson Disease (PD) and another had Lewy Body Dementia (LBD).
The control group included a population with an average age of 71.3±5.4 years (n=10, 5 men and 5 women) an were form.
The urine was collected over the night and out of light. Urine samples were stored at –80ºC until analysis. Before analysis, all urine samples were centrifuged and filtered as described above.

The analytical methodology was the same as previously described in Chapter 3 (for DMPN and DMPN NAC) and Chapter 5 (for 2,5-HD).
In brief, a rapid and easy urinary pyrroles detection (Ehrlich's reagent) followed by identification of 2,5-HD, DMPN and DMPN NAC by LC-MS/MS were performed.

However, due to unexpected problems (disassembly of the LC-MS/MS system, as a consequence of rehabilitation works in the building and a fire in the nearby laboratory) was not possible to quantify the target analytes.

6.3 RESULTS

The presence of pyrrole compounds was detected in all analyzed urines using Ehrlich's reagent by a spectrophotometric method.

6.3.1 IDENTIFICATION OF 2,5-HD AND PYRROLE COMPOUNDS BY LC-MS/MS

After urines pretreatment, all samples were analyzed by LC-MS/MS.

The ESI-LC-MS/MS analytical conditions described previously in Chapter 3 and in Chapter 5, allowed us to identify DMPN, DMPN NAC and 2,5-HD in urine of patients and healthy subjects.

Table 6.2 Conditions obtained for studied compounds (see Chapter 3 and 5).

Rt (min)	Compound	Precursor ion (*m/z*), $[M+H]^+$	Product ions, *m/z* (relative intensity)	*MRM*1 transition	*MRM*2 transition
12.4	**2,5-HD**	115	43(76), 55(19), **69**(46), 79(27), **97**(100)	115>97	115>69
11.1	**DMPN NAC**	428	**410**(12), 351(19), **299**(100), 297(36), 194(6)	428>299	428>410
13.6	**DMPN**	267	**225**(19), 180(37), **162**(100), 108(37), 96(65)	267>162	267>225
18.7	**2,5-DMP**	96	96(100), 95(37), **80**(73), **81**(40),79(15), 68(36)	96>80	96>81

The same analytical conditions described previously were used to detected 2,5-HD and 2,5-DMP in standard solutions (100 ppm). These were performed by spiking pooled human healthy urine with 2,5-HD or 2,5-DMP.

As can be seen in the chromatograms (Fig. 6.1 A and B), 2,5-HD is detected at Rt=11.7 min and 2,5-DMP is detected at Rt=18.1 min.

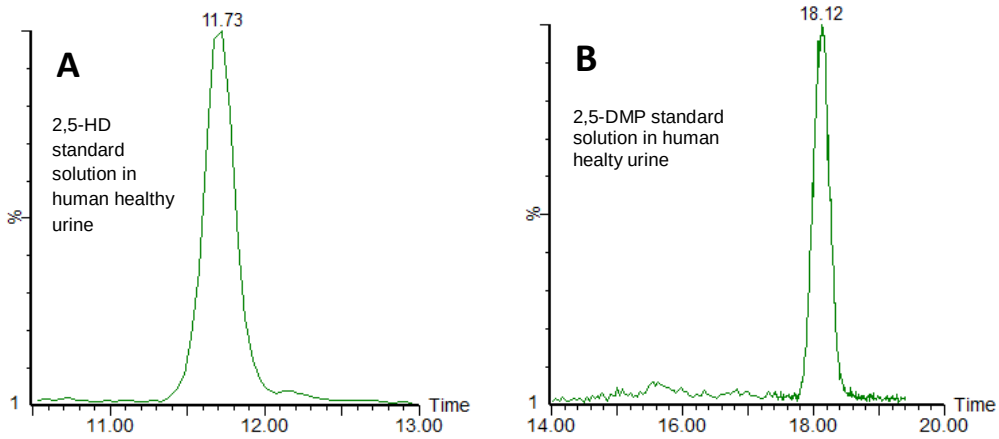


Figure 6.1 Chromatograms in MRM mode for standard solutions (100 ppm) of 2,5-HD (A) and 2,5-DMP (B). TIC obtained for the most important transitions for 2,5-HD and 2,5-DMP, in human healthy urine.

Chromatograms profile corresponding to 2,5-HD detected in urines are presented in Figure 6.2.

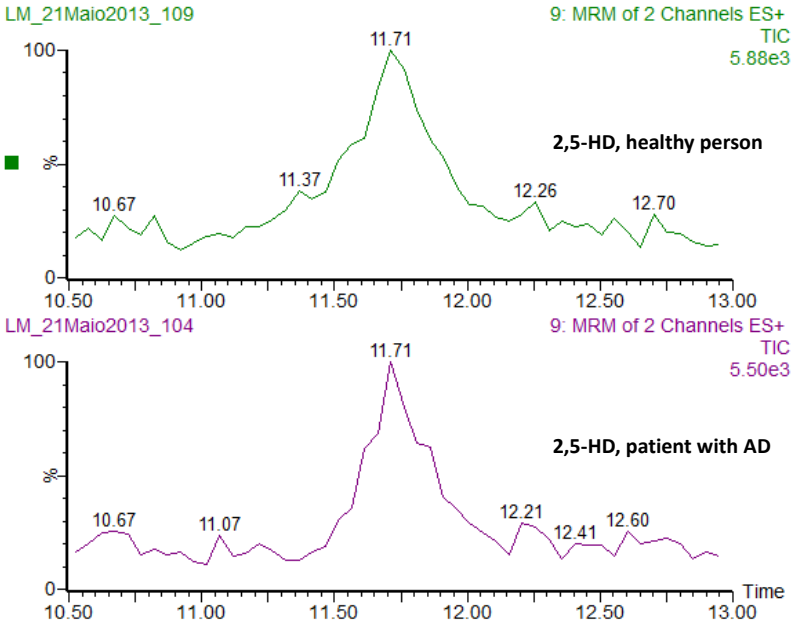


Figure 6.2 Chromatograms in MRM mode for 2,5-HD. TIC obtained for the most important transitions for 2,5-HD: *MRM*1, 115>97 and *MRM*2, 115>69. Detection in urines of healthy person and patient with Alzheimer Disease (AD).

These chromatograms were obtained in MRM mode of the two most important transitions for 2,5-HD detected in urine: *MRM*1, 115>97 (transition of quantification) and *MRM*2, 115>69

(transition of confirmation). In this figure are presented two of the MRM chromatograms of 2,5-HD identified in the urines of one healthy person and one patient with Alzheimer Disease (AD). The 2,5-HD in urine of rat had a Rt=12.4 min, however in human urine has a Rt=11.7 min. There was a decrease in the retention time (less 0.7 min).

The same analytical conditions described previously in Chapter 3, were applied to the identification of pyrrole compounds in urine of patients and healthy subjects.

For analytical conditions used, the compound 2,5-DMP was not detected in the urine of patients and healthy people.

However, for identification of DMPN, compound at *m/z* 267, chromatograms were obtained, in MRM mode choosing the most important transitions 267>162 (*MRM*1) and 267>225 (*MRM*2) (Figs. 6.3 and 6.4).

In Figure 6.3 are presented chromatograms in MRM mode of urine of patients: one with Alzheimer Disease and two with Vascular Dementia (VD).

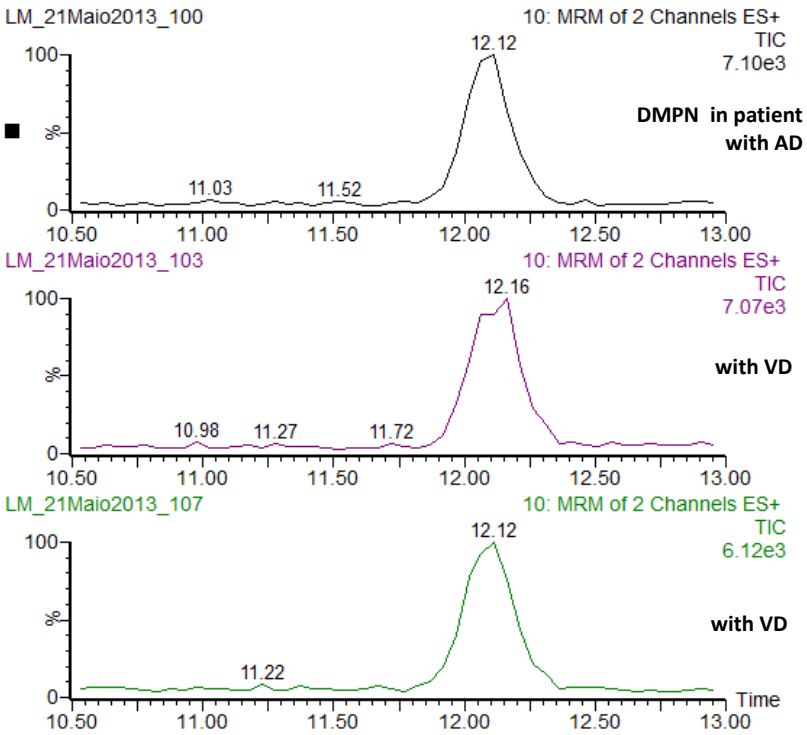


Figure 6.3 Chromatograms in MRM mode for DMPN, detected in urines of patients with Alzheimer Disease and with Vascular Dementia. TIC obtained for the most important transitions: 267>162 (*MRM*1) and 267>225 (*MRM*2).

Chromatograms in MRM mode, selecting the same transitions *MRM*1 and *MRM*2, to identify DMPN in urines of healthy people are presented in Figure 6.3.

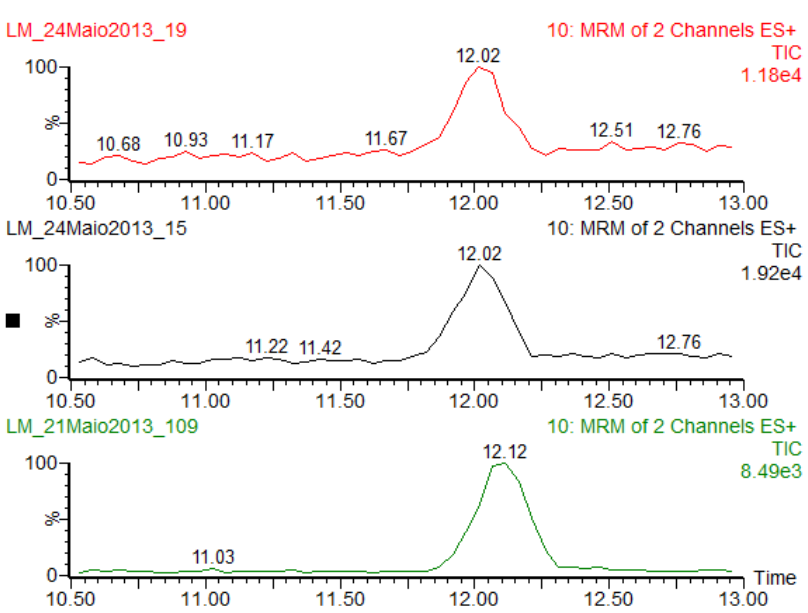


Figure 6.4 Chromatograms in MRM mode for DMPN, detected in urines of healthy people. TIC obtained for the most important transitions 267>162 (*MRM*1) and 267>225 (*MRM*2).

The pyrrole compound, DMPN, in urine of rat had a Rt=13.6 min, however in human urine has a Rt=12.1 min. There was also a decrease in the retention time (less 1.5 min).

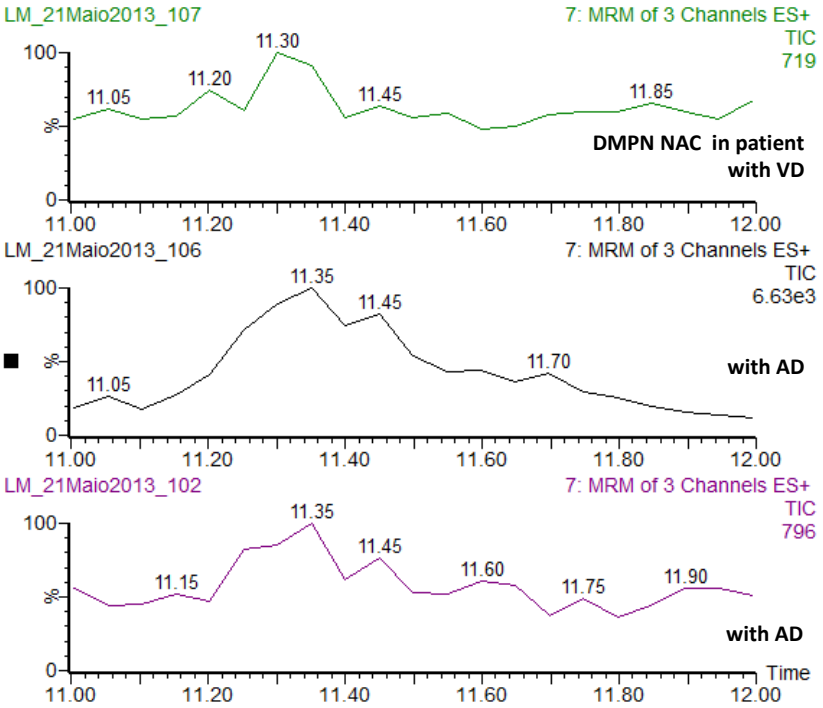


Figure 6.5 Chromatograms in MRM mode for DMPN NAC, detected in urines of patients with VD and with AD. TIC obtained for the most important transitions 428>299 (*MRM*1) and 428>410 (*MRM*2).

The precursor ion $[M+H]^+$ at *m/z* 428 of DMPN NAC originated fragments *m/z* 299 and *m/z* 410 (Chapter 3). Chromatograms were obtained, in MRM mode choosing the most important transitions 428>299 (*MRM*1) and 428>410 (*MRM*2) (Fig. 6.5). However as can be see through the chromatograms only trace amounts of DMPN NAC were found. For this compound the variation of Rt was minimal (more 0.2 min).

In a pH balanced human body, urine is slightly acid in the morning, (pH = 6.5 - 7.0). However, generally becomes more alkaline (pH = 7.5 - 8.0) by evening in (healthy) people primarily because no food or beverages are consumed while sleeping. During the day the body buffers the pH of the food and beverages consumed by releasing electrolytes and the pH level goes up.
To facilitate the procedure for collecting the urines of patients, they were collected overnight. For rats, urine pH range is 5-7, and urine is collected for 24 h.

This difference between the pH of human urine and the pH of the urine of rats may perhaps explain the difference of retention times.

This difference, which is found between the various retention times, may be related to ionization of the compounds in human urine.
In this case, the basic pH of human urine will cause, in the more ionizable compounds, 2,5-HD and DMPN, a shift of peak area and in human urine these compounds exhibit a lower Rt.

In conclusion, in all the analyzed urines (9 +10), was possible to identify 2,5-HD and DMPN, and in all patients urines (9) was also identified trace amounts of DMPN NAC.

6.4 DISCUSSION AND CONCLUSION

The objective of this preliminary human study was to apply the studied biomarkers in two aged populations: one healthy (Group I) and one sick with neurodegenerative diseases (Group II).
Our first results show that 2,5-HD and the pyrrole compound DMPN were identified in the urine of all healthy and patients individuals (Figs. 6.2 to 6.4) and, in urine of patients was also identified DMPN NAC (Fig. 6.5).

The first step of this study was to confirm the presence of total urinary pyrroles in the 2 groups. The Ehrlich's reaction was found positive in all the urines and these results are in accordance with others authors which refer the presence of pyrroles in not n-hexane exposed populations (Kessler et al., 1990; Ogata et al., 1991).

Subsequently was applied LC-MS/MS analysis, to search for the studied target analytes: urinary 2,5-HD and the previously identified pyrrole compounds DMPN and DMPN NAC.
Figures 6.2, 6.3 and 6.4, show that DMPN and 2,5-HD were detected in both Groups of Humans (I and II).

The presence of 2,5-HD in urines may be explained by the involuntary exposure to n-hexane by the general population, as is an ubiquitous micropollutant, and by the endogenous production of n-hexane and 2,5-HD, through reactions of metabolism and/or lipid peroxidation in human body as referred by several authors (Fedtke and Bolt 1986a; Perbellini et al., 1986, 1993; Brugnone et

al., 1991; Bavazanno et al., 1998; Tondel et al., 2011). Both hypothesis might explain the appearance of levels of 2,5-HD in all analyzed urines.

Concerning, the urinary DMPN identified in all the urines, its origin is probably due to the endogenous 2,5-HD production and its subsequent reaction with amino groups of lysine. Certainly, we need to perform a new study with a larger healthy population, where it is quantified 2,5-HD and DMPN to confirm these preliminary results.

The other target pyrrole, DMPN NAC, was not detected in control urines. This is an interesting result considering that also in rats control and not co-treated with NAC was not possible to quantify DMPN NAC. However, in all nine patient urines DMPN NAC was detected.
Obviously, it is very difficult to interpret the source of DMPN NAC found in a few samples of patients with different neurodegenerative diseases.

Human brain studies suggest that oxidative stress has an important role in neuronal degeneration in elder patients and a convergence of several factors (genetic, age related and environmental) initiate a common pathophysiological cascade involving oxidative stress, lipid peroxidation and the subsequent generation of products in disease-specific brain regions (LoPachin et al., 2008a,b): *e.g.*, the hippocampus and neocortex of AD brain in these patients.
Thus, the increase of electrophilic products resulting from lipid peroxidation in elder individuals could produce synaptotoxicity through their adduction to protein nucleophilic with sulfhydryl groups which have the role of intermediate mediators of synaptotoxicity in many neurodegenerative diseases.
In conclusion, it is likely, that in part the urinary DMPN and DMPN NAC, detected in patients may be due to the increase formation of pyrrole adducts with amino and sulfhydryl groups in proteins which may contribute to the neurological effects observed in neurodegenerative diseases.

ANNEX I:

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorrecto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Título do estudo:

Desenvolvimento e validação de biomarcadores para avaliação e prevenção de efeitos neurotóxicos

Enquadramento:

Trabalho a realizar na Faculdade de Farmácia da Universidade de Lisboa

Explicação do estudo:

Este estudo enquadra-se numa parte do doutoramento correspondente à recolha de urinas de pacientes de idade avançada com doenças neurodegenerativas. A recolha e o estudo dessas urinas irá permitir fazer um estudo no âmbito de biomarcadores preditivos de efeitos neurotóxicos, uma vez que podem ser considerados uma importante ferramenta para a prevenção e promoção da saúde pública.

A caracterização de determinados compostos (derivados pirrólicos) e o estudo do mecanismo de interacção com agentes antagonistas ou protectores da formação dos aductos pirrólicos poderá ajudar a esclarecer o mecanismo de protecção ou antagonismo que reduz o desenvolvimento de alterações moleculares e celulares e eventualmente identificar a origem de algumas doenças neurológicas de origem desconhecida. Nesta faixa etária as doenças neurodegenerativas representam um factor importante no agravamento das condições de saúde. Assim, a prevenção destas doenças através do controlo e prevenção da exposição a xenobióticos neurotóxicos e/ou indutores do stress oxidativo, contribuirá decisivamente para um melhoramento da qualidade de vida das populações expostas ou geneticamente predispostas ao seu desenvolvimento.
Após a recolha e análise das urinas dos pacientes, as respectivas urinas serão destruídas.

Confidencialidade e anonimato:
Garante-se a total confidencialidade e o uso exclusivo dos dados recolhidos para o presente estudo.

Expressamos o nosso reconhecimento pela sua disponibilidade em colaborar.

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Assinaturas:

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CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis is inserted in the area of Toxicology, and is a contribution to the selection of specific biomarkers to be applied in control and prevention of neurotoxicity in risky populations.

An analytical and one toxicological approach were used to develop this research project:

In **Chapter 3** an analytical approach was applied to study alternative biomarkers of n-hexane exposure.

To achieve this goal we performed:

the synthesis of standard pyrrole compounds,

the identification of DMPN, DPMN NAC and DMPN GSH in standards and in urine of rats treated with 2,5-HD by LC-MS/MS,

the quantification of DMPN and DMPN NAC in rats treated with 3 doses of 2,5-HD by the same method.

In **Chapter 4** and **Chapter 5** a toxicological approach was used to investigate if the identified pyrroles could be selected as biomarkers of 2,5-HD neurotoxicity and to clarify the role of NAC in its toxicity.

To achieve this goal were performed 2 *in vivo* assays with repeated dose treatments of 2,5-HD and 2,5-HD+NAC.

- In **Chapter 4** was evaluated the correlation between 2,5-HD, DMPN and DMPN NAC urinary levels and the motor activity performance in rats treated with 4 doses of 2,5-HD and co-treated with 4 doses of 2,5-HD+NAC.
- In **Chapter 5** the protection *versus* toxicity effects of NAC on 2,5-HD neurotoxicity was investigated increasing the number of administrated doses. Along the four weeks assay, motor activity performance, 2,5-HD, DMPN and DMPN NAC levels were evaluated, and after rats' sacrifice, brain GSH and Cys levels were determined in all rats.

Finally, in **Chapter 6** a preliminary study was performed in human urine of an healthy and a sick population to investigate the presence of the identified pyrroles.

The results obtained in the experimental work of this thesis allowed us to synthesize the following final conclusions:

FINAL CONCLUSIONS

- DMPN, DMPN NAC and DMPN GSH were identified, for the first time by LC-MS/MS, in urine of rats treated with 2,5-HD.

- In rats treated with 2,5-HD were quantified DMPN and DMPN NAC being DMPN presented in much higher levels than DMPN NAC in all analyzed urines, thus, DMPN may be suggested as an specific and sensitive biomarker of 2,5-HD exposure.

- NAC decreases the excretion of urinary levels of 2,5-HD and DMPN and increases the excretion of DMPN NAC in rats co-treated with 2,5-HD+NAC as compared with their levels in rats treated with 4 doses of 2,5-HD alone.

- NAC interferes on the reduction of motor activity performance observed in rats treated with 2,5-HD: until the 4 doses of co-treatment, NAC promotes the recovery of rearing and ambulation, with a progressive attenuation of motor activities recovery after 8 and 12 doses of co-treatment.

- The mechanism of NAC interference on 2,5-HD neurotoxicity may be suggested as a biphasic process:
    i) along the first 4 doses of co-treatment, a significant reduction of urinary free 2,5-HD and DMPN levels may be an indicator of DMPN decrease formation in axonium proteins, decreasing the probability of neurofilaments crosslinking which could explain the observed recovery of motor activities;
    ii) after the 4 doses co-treatment a progressive attenuation of motor activity recovery was observed with decrease of DMPN excretion. This fact probably induces an accumulation of DMPN in axonium proteins with the subsequent increase of motor activity dysfunctions.

- The increase of DMPN NAC along the study in treated and co-treated rats, suggests that this pyrrole is a specific biomarker of 2,5-HD exposure. However, this compound is excreted in much lower levels than DMPN.

- The well known persistence of DMPN NAC adducts, indicates that along the experiment, this aminopyrrole thiol conjugate may accumulate in axonal cytoskeletal proteins which in part contributes to the progressive increase of neurobehavior dysfunctions.

- Finally the first results obtained in human urine of healthy and sick individuals suggest that DMPN may be detected in human urine in elder healthy and sick populations.

FUTURE PERSPECTIVES

In vivo models with Wistar rats at a longer period (8 weeks) of co-treatment with 2,5-HD+NAC to determine DMPN and DMPN NAC in urine, erythrocytes, sciatic nerve and brain.

New battery of behavioral assays to accurately correlate the levels of determined pyrroles in biological samples with the changes in behavioral performance (for each dose).

In vitro models to evaluate the mechanism of interaction of NAC with 2,5-HD using the human neuroblastoma line SK-N-SH to clarify the NAC threshold levels in protecting against 2,5-HD neurotoxicity.

Identification and quantification of DMPN, DMPN NAC, and other pyrroles namely kripto-pyrroles in urine of a larger group of individuals, patients (environmental or occupational exposed), with neurodegenerative diseases and in healthy individuals.

ANNEX

STUDY OF STABILITY OF 2,5-DMP AND DMPN IN RAT URINE

ANNEX

STUDY OF STABILITY OF 2,5-DMP AND DMPN IN RAT URINE

Before identifying the different pyrroles in urine we performed a preliminary study to investigate their stability in urine.

Urine of animals of first *in vivo* study, rats treated with 3 doses of 2,5-HD.

In the presence of oxidizing agents, or on account of their low stability, 2,5-DMP, may lead to formation of dimers and trimmers. Although they have not been synthesized, its presence was confirmed by the Daughters Scans of its precursor ions.

MS/MS spectra of dimer and trimmer of 2,5-DMP

In Figure 1 are shown the 2,5-DMP structures, dimer and trimmer. The molecular form and molecular weight is $C_{12}H_{16}N_2$ and 188.27 g/mol for dimer, and $C_{18}H_{23}N_3$ and 281.40 g/mol for trimmer.

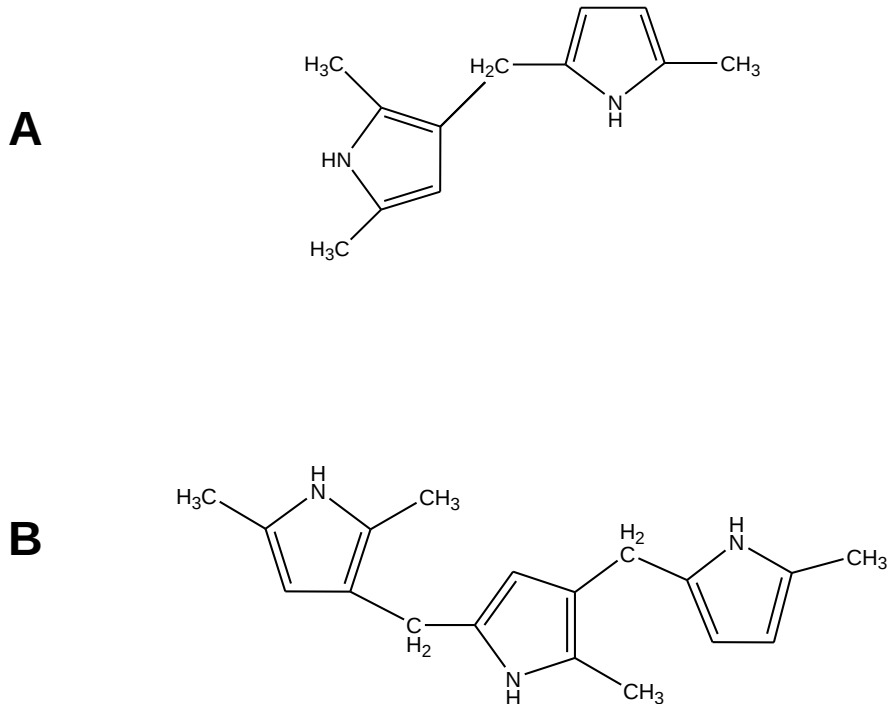

Figure 1. Structures of dimer (A) and trimmer (B) of 2,5-DMP.

In Figure 2 (A and B) is shown the fragmentation of the precursor ion $[M+H]^+$ at *m/z* corresponding to dimer and trimmer of pyrrole compound. In these mass spectra are presented the daughters scans of precursor ion *m/z* 189 for dimer (A) and the daughter scans of precursor ion *m/z* 282 for trimmer (B).

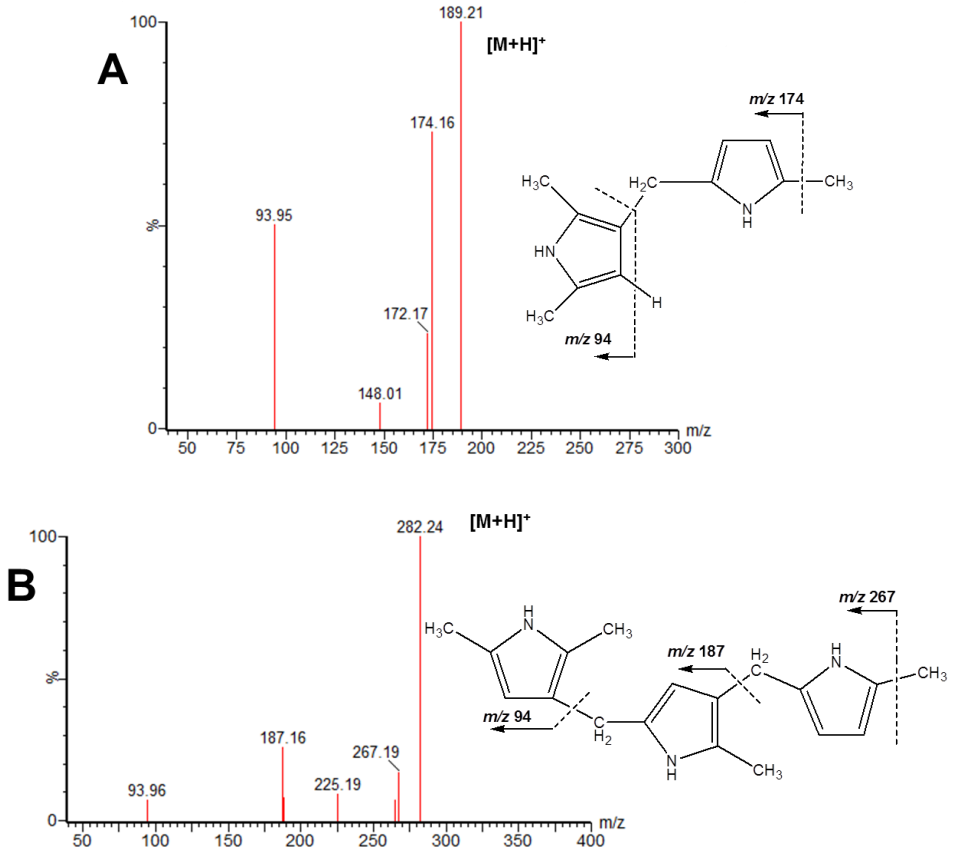

Figure 2. MS/MS spectra of dimer and trimmer of the standard pyrrole compound, 2,5-DMP, obtained after the fragmentation of the molecular ion (*m/z* 189) (**A)** and (*m/z* 282) (**B)**.

Fragmentation of dimer and trimmer of 2,5-DMP at *m/z* 189 and *m/z* 282 originated several fragments, some common. For 2,5-DMP dimer, an ion fragment at *m/z* 174 corresponding to the loss of methyl group $[M{-}CH_3)]^+$, another at *m/z* 94 corresponding to the 2,5-dimethylpyrrole fragment. For precursor ion *m/z* 282, an ion fragment at *m/z* 267 corresponding to the loss of

methyl group $[M{-}CH_3)]^+$, another at *m/z* 187 corresponding to the fragment of dimer of 2,5-dimethylpyrrol and at *m/z* 94 corresponding to the 2,5-dimethylpyrrole fragment.
These compounds, dimers and trimmers of 2,5-DMP were detected in urine however were not quantified.

Standard 2,5-DMP Solution in Control Urine

The stability of 2,5-DMP in urine matrix was studied knowing that this compound easily undergoes dimerization and trimmerization reactions (Zhu et al., 1995, 1997).

Thus, we used a pool of urines of control rats added of 2,5-DMP standard solution which were analyzed over time by LC-MS/MS. In Figure 3 is shown the TIC chromatogram of 2,5-DMP with a concentration of 100 ppm in a pool of urine of a control group (II) of rats. It was found that after 32 h, the peak of 2,5-DMP (Rt~18.7 min) decreases nearly for half height.

In Figure 4 it is observed the overlapping of the peaks resulting from the analysis of the test solution, over the time. It is noted a significant decrease in the peak with Rt=18.67 minutes and an increase of the peak at Rt=15.66 min. No change in signal intensity, relative to the compound with a retention time at Rt=13.54 min, was found.

The decrease of 2,5-DMP was also tested with other urine pools using a lower concentration of 2,5-DMP solution. After 32 hours, for a 10 ppm solution of 2,5-DMP in a pool of urine rats of group II a decrease of ~50% in 2,5-DMP concentration was found.

In Figure 5 it is shown the peaks of the 10 ppm 2,5-DMP solution, under N_2 atmosphere, over the time. It is noted a relative decrease in the peak with Rt~18.6 minutes, and an increase of the peak at Rt~15.5 min. There seems no change in signal intensity, relative to the compound with a retention time at 13.9 min.

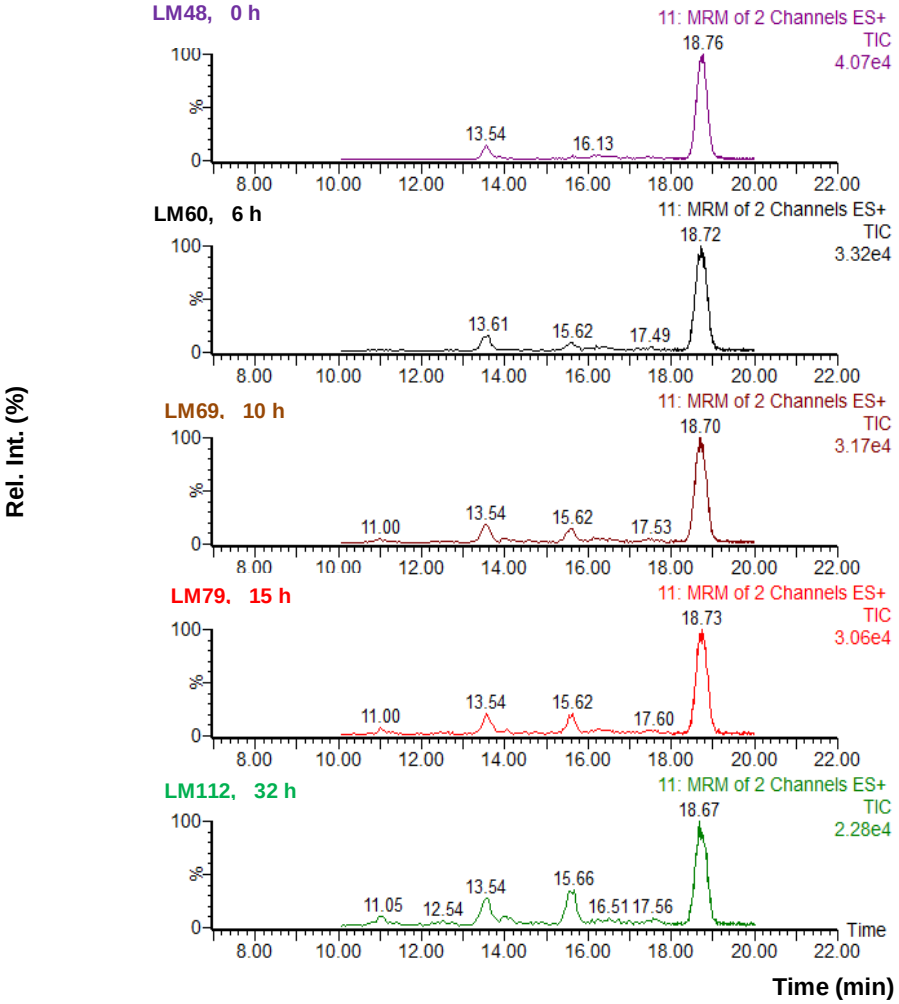

Figure 3. TIC chromatograms of pyrrole compound with a concentration of 100 ppm in a pool of urine of a group of control rats, over the time.

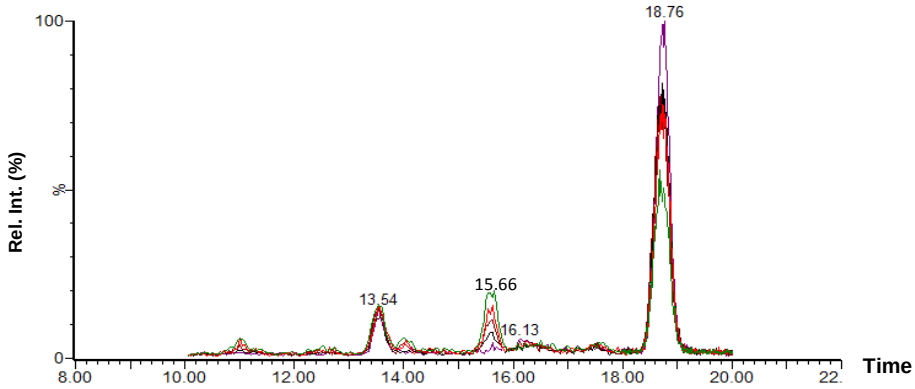

Figure 4. Overlapping of the peaks resulting from TIC chromatograms of pyrrole compound with a concentration of 100 ppm in a pool of urine of a group of control rats, over the time.

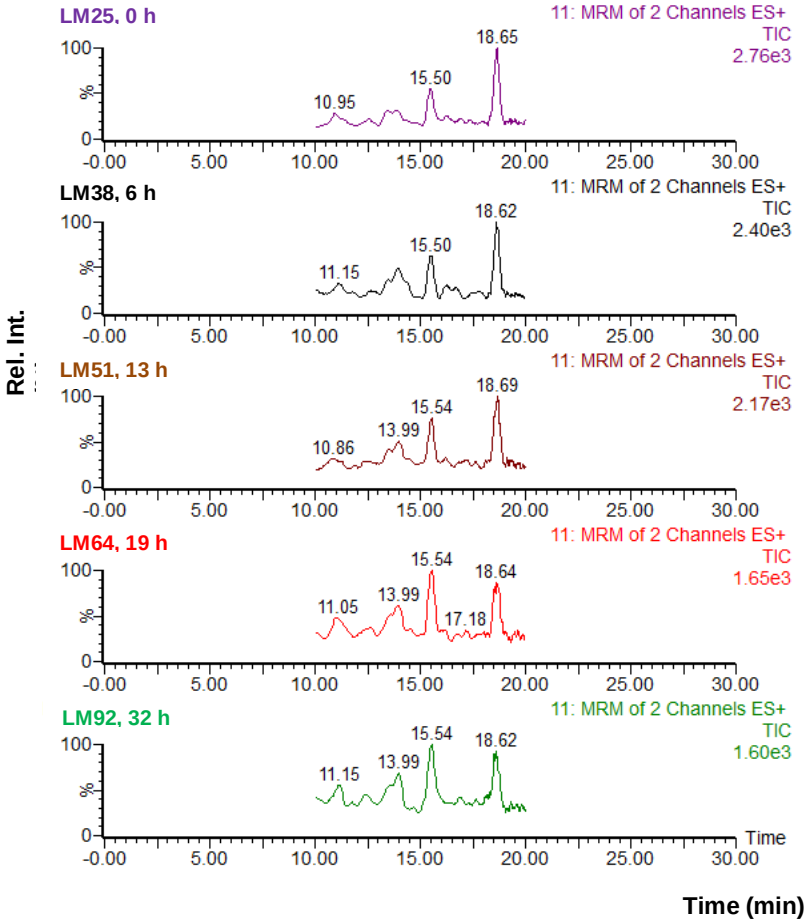

Figure 5. TIC chromatograms of pyrrole compound with a concentration of 10 ppm in 2,5-DMP (in N_2) in a pool of urine of a group II of rats, over the time.

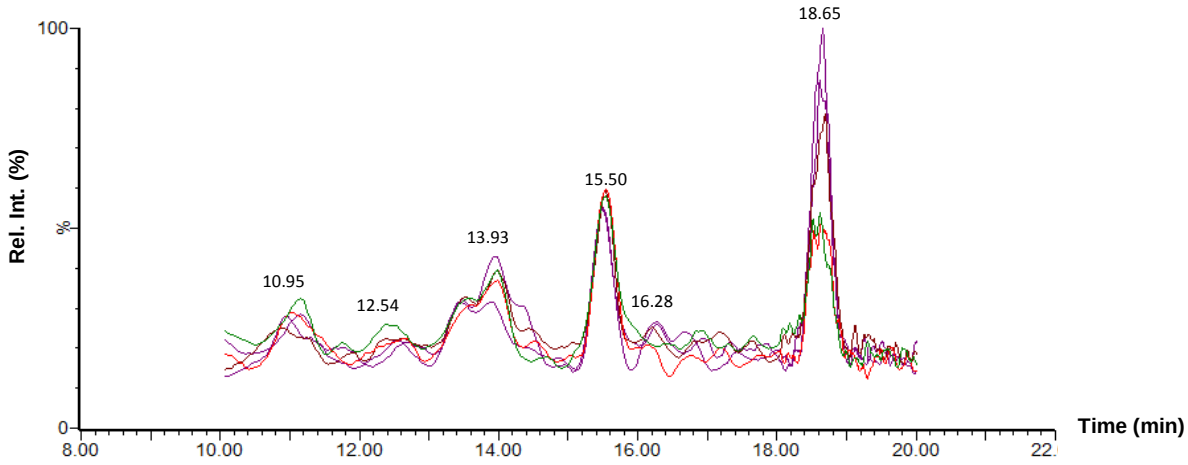

Figure 6. Overlapping of the peaks resulting from TIC chromatograms of pyrrole compound with a concentration of 10 ppm of 2,5-DMP, under N_2 atmosphere, in a pool of urine of a group of control rats over the time.

In Figure 6 we can observe the overlapping of the peaks, over the time. Now, it can be seen a significant decrease in the band with Rt~18.6 minutes. The band at Rt~15.5 min there seems no change in signal intensity.

DMPN in Urine of Exposed Rat

Unlike the compound 2,5-DMP, the pyrrole DMPN presents itself as a stable compound over time. The study was conducted using a pool of urines of rats exposed to 3 doses (400 mg 2,5-HD/kg/48-h) which was analyzed by LC-MS/MS.

In Figure 7 is shown the TIC chromatogram of DMPN in a pool of urine of rats of Group I (for 3 doses). It was found that after 48 h, the peak of DMPN (Rt~13,7 min) don't decreases and continues to present the same intensity presented at time 0 h.

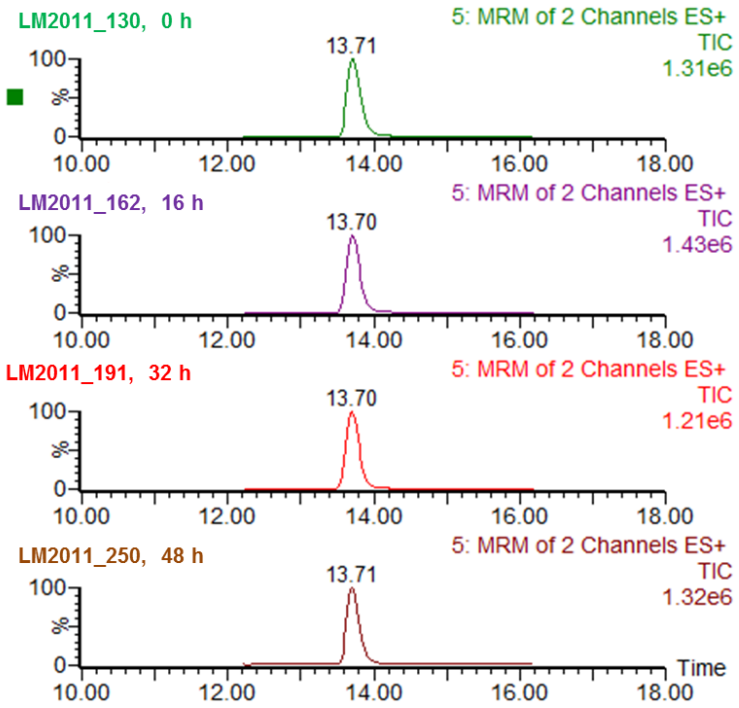

Figure 7. TIC chromatograms of pyrrole compound DMPN in a pool of urine of group I of rats, over time.

Conclusion:

Unlike the compound 2,5-DMP, the pyrrole DMPN presents itself as a stable compound over time.

It is shown the peaks of the 10 ppm DMP solution, under N_2 atmosphere, over the time. It is noted a relative decrease in the peak with Rt~18.6 minutes, and an increase of the peak at Rt~15.5 min (Fig. 4). Even under N_2 atmosphere, DMP may form others compounds, probably dimmer and/or trimmers.

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